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Astrocyte effects on hippocampal synaptogenesis in culture and near-field microscopy

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Astrocyte effects on hippocampal synaptogenesis in culture and near-field microscopy

by

Robert Thomas Doyle

A dissertation submitted to the graduate faculty

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DOCTOR OF PHILOSOPHY

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For the Graduate College

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"What is the function of the neuroglia in the nervous system? At the present, no one knows, but even worse still is the fact the this problem has remained unsolved for a long time because the physiologists lack direct methods to study this phenomenon." (Santiago Ramón y Cajal, The Neuron and the Glial Cell).

CHAPTER 1. GENERAL INTRODUCTION

The focus of this dissertation is twofold: first, the affect of astrocytes on synapse formation and second, the presentation of a new imaging technique for high-resolution fluorescence investigations at and just below the membrane of cells. The first paper addresses the concept that astrocytes play a part in forming the synaptic connections between neurons and adds to the emerging picture that glial cells are active participants in brain function. The second paper introduces a near-field imaging technique that has been designed for use in biological investigations

The key to neural functioning is centered on communication among the cells that comprise the nervous system. It has been established that the key to this communication is the specialized contact that exists between nerve cells for information transfer, the synapse.

The Neuron Doctrine

Before the concept of the synapse was espoused by Sherrington (Shepard, 1988) in 1897, the Neuron Doctrine had displaced the reticular theory on how the nervous system functioned and placed neurons at the epicenter of brain function. The reticular theory was entrenched in the mid to late 1800's (Dierig, 1994; Edwards and Huntford, 1998; Katz-

Sidlow, 1998) and held that the nervous system was a continuous network of cellular material.

Making use of an advance in microscopy, Nansen (Edwards and Huntford, 1998) examined the nervous tissue of invertebrates with oil immersion lenses and published the first paper calling the basic concept of the reticular theory into question, he had seen free nerve endings. Studies by others appeared almost at the same time (Edwards and Huntford, 1998) and set the stage for a change in thinking about the nervous system (Fodstad et al., 2000). The term 'neuron' was introduced in 1891 by Waldeyer (Dierig, 1994; Edwards and Huntford, 1998; Katz-Sidlow, 1998) in an article reviewing the work of six researchers, Nansen, His, Koelliker, Lenhossek, Ramón y Cajal, and Retzius, all of whom had contributed data disputing the reticular theory. Waldeyer wrote that their ideas could be summarized into a concept that the nervous system is composed of *"innumerable units, not connected genetically or anatomically"* (Edwards and Huntford, 1998). Waldeyer's summary provided the basis for new scientific dogma, the Neuron Doctrine. From that point on, the nervous system was only seen as the sum of the capabilities of neurons.

The Subservience of Glia

Glial cell research had reached a prolific point at the same time that the Neuron Doctrine emerged. Glia were also recognized as distinct entities and as part of the architecture of the brain but not as functional units of information processing. They were looked upon by their discoverer, Rudolph Virchow, as the 'glue' (nervenkitt) that holds the neurons together(Dierig, 1994; Finger, 2000). The view that these cells were nothing more than passive comrades of nerve cells remained the view of most neurobiologists.

The fact that glial cells existed to serve the needs of neurons was only strengthened as new data describing these types of functions were presented. Glia are responsible for maintenance of homeostasis of the neuronal environment, they serve as a nutritional conduit from endothelial cells to neurons (Poitry-Yamate et al., 1995) (Tsacopoulos and Magistretti, 1996) , and act as a supplier of metabolic precursors needed by the neurons to produce neurotransmitters (Laake et al., 1995; Lapidot and Gopher, 1994; Westergaard et al., 1995). Neurons were found to be electrically excitable and advances in the technology used to detect electrical signals propelled neurons to a place of prominence in brain function. By their electrical silence, glial cells were ignored when investigations of information processing in the CNS were considered.

Dogma and Change

Change in understanding how physiological processes work, once dogma has been accepted, can be extremely difficult. The brain itself was overlooked for hundreds of years and it took Hippocrates to change the view of the heart as the center of perception to the view that the brain was the seat of "*...joys, delights, laughter and sports, and sorrows, griefs, despondency and lamentations*" (Finger, 2000). The power of dogma is again exemplified in the existence of the "rete mirabile", a structure of blood supply at the base of the brain thought to be responsible for the generation of 'animal spirits'. This structure 'existed' in human anatomy for over 1300 years because Galen said so. Galen used animals as models for his dissections and studies of anatomy and this structure is indeed found in the species that he actually dissected. Galen assigned this structure to the human body to explain the functioning of the brain and this fact was not questioned until the early 1300's by Mondino de' Luzzi.

However, it took until the 1500's when Versalius corrected the "mistake". Versalius, trained in the methods of Galen, lectured on the existence of the "rete mirabile" in man and even demonstrated it during public dissections. He may have become distressed by the fact that he always needed a sheep's head available during these demonstrations to actually show the structure and finally denied its existence in humans in his landmark *De humani corporis fabrica* (Finger, 2000).

Glial cells enjoyed a subservient position relative to neurons based on what was observed to be their function. This view became dogma even though we had been forewarned. Future neuroscientists were admonished by Ramón y Cajal in (The Neuron and the Glial Cell) not to overlook the capacity of these cells:

> This is truly a prejudgement, in that the neuroglial cells in the same manner as the collagenous bundles of the connective tissue act in relation to the musclar or granular cell. We accept this relationship as though it were a solid established fact that these fibrils form a passive support to simply fill out and bind the tissue in a matrix that is swelled with nutritive substance. **Every investigator who wishes to form a rational opinion concerning the activity of neuroglial cells should abandon this manner of thinking which predisposes this judgement.** (emphasis mine p.282)

Advances in Science Are Tied to Advances in Technology

Advances in physiological research are tied to advances in the experimental techniques used to perform the research. A quote from Edgar Adrian's 1932 Nobel Prize Address (Finger, 2000) will illuminate this point:

> The nerves do their work economically without visible change and with the smallest expenditure of energy. The signals which they transmit can only be detected as changes of electrical potential, and of very brief duration. It is little wonder therefore, that progress in this branch of physiology has always been governed by the progress of physical technique and that the advent of the triode valve (three stage) amplifier has opened up new lines in this, as in so many other fields of research. (p. 252)

The development and advances in monitors of intercellular ion concentration ushered in a new field of cellular physiological investigation. The development of fluorescent indicators (Grynkiewicz et al., 1985; Poenie and Tsien, 1986; Tsien, 1988; Tsien and Harootunian, 1990) to report ion concentration and location allowed researchers to ask questions that they had not been able to in the past. With these technical advances, studies show that glial cells are emerging as much more complex entities than had been appreciated. In the early 1990's, researchers discovered that glial cells were 'calcium excitable' and communicate via calcium signaling (Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; MacVicar, 1984; Nedergaard, 1994; Smith, 1992). First, this communication was established in cell culture ((Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Cornell-Bell et al., 1990; Finkbeiner, 1992; Finkbeiner, 1993) and then in hippocampal (Dani et al., 1991; Dani et al., 1990; Dani et al., 1992; Dani and Smith, 1995) and retinal slices (Newman and Zahs, 1998).

It was later discovered that glia also release neurotransmitters and influence neuronal function (Araque et al., 1998a; Araque et al., 1999a; Araque et al., 1998b; Araque et al., 1999b; Parpura et al., 1994; Parpura et al., 1995a; Parpura et al., 1995b; Sanzgiri et al.,

1999). An important advance was demonstrated when it was shown that physiological changes in calcium levels in astrocytes (the main class of glial cells in the brain) were sufficient to cause neurotransmitter release (Parpura and Haydon, 2000). Additionaly, astrocytes express proteins that are involved in neurotransmitter release (Jeftinija et al., 1997; Parpura et al., 1995a) and utilized a calcium-dependent mechanism reminiscent of the release process utilized by neurons (Araque et al., 2000).

In light of this new data relative to the capabilities of glia, a change in our thinking about how synapses function is taking place (Araque et al., 1999a; Haydon, 2001; Smith, 1992; Smith, 1994; Smith, 1998; Verderio et al., 1999; Vesce et al., 1999). It is clear from these studies that glial cells are an intricate element in neuronal function. A natural question follows: if glia are found to be an integral part of the physiology of the mature brain, do they also play a fundamental role in forming the connections that are the basis of brain function – the synapses?

The Synapse

The synapse is a morphologically specialized asymmetric cell-cell contact where information passes from a neuron to its target cell. In the CNS, synapses are found between neurons and are morphologically characterized by tight apposition of the pre- and post-synaptic membranes accompanied by specializations in both cells that serve to accomplish neurotransmitter release (presynaptic) and reception (postsynaptic). The presynaptic terminal of the synapse is further characterized by an accumulation of small synaptic vesicles which contain neurotransmitter. A portion of these vesicles are found aligned along the presynaptic membrane with the majority of the vesicles located some distance from the membrane.

The physiological concept of the synapse was introduced in 1897 by Sir Charles Sherrington in chapters to a revision of a standard physiology text by Michael Foster (Shepard, 1988). Sherrington presented the concept in this proposal: *"So far as our present knowledge goes, we are led to think that the tip of a twig of the arborescence is not continuous with but merely in contact on which it impinges. Such a special connection of one nerve cell with another might be called a synapse."* Sherrington was not able to see the synapse in the pre-electron microscope days, but he surmised that the structure had to exist to explain the physiological data that had been collected in studies on reflex action and excitatory and inhibitory effects observed in experiments (Eccles and W.C., 1979; Finger, 2000). Now advances in technology have allowed progress in our understanding of how the brain functions. These advances, in turn have opened the door on more questions. One question at hand is: Do astrocytes influence synapse formation? It is indeed curious that Sherrington, who gave us the term 'synapse', was once a student of Virchow, the discoverer of the glial cell. Now we see a confluence of scientific concepts introduced by these great scientists in the idea that astrocytes influence the formation of the synapse.

This question has been approached very few times. However, the possibility that astrocytes could influence synapse formation seems quite logical. Neurons respond to glial-derived growth factors and cytokines with a spectrum of responses, ranging from survival to plasticity. However, the fact that glial cells can greatly influence the health of neurons becomes a large impediment to conducting experiments without the presence of glia. In 1994 Nakanishi et. al. (Nakanishi et al., 1994) used calcium imaging to present the possibility that synapse formation in rat cortical culture required astrocytes that were site and age specific. This study did not utilize electrophysiology to confirm synaptic function. In 1996 two

laboratories presented preliminary results (Doyle et al., 1996; Pfrieger and Barres, 1996) that astrocytes significantly influences synapse formation in embryonic rat hippocampal and postnatal rat retinal ganglionic cultures by conducting experiments in glia-containing conditions and glia-deplete or 'glia-free' conditions . Pfrieger and Barres reported that astrocytes enhanced the efficacy of synapse formation in postnatal rat retinal cultures in 1997 (Pfrieger and Barres, 1997). They utilized an immunopanning technique to produce neuronal cultures that were 99.5% pure and essentially glia-free. They reported that developing neurons in culture form inefficient synaptic connections that require glia cells to become fully functional. The Barres laboratory extended this research and reported in 2001 (Ullian et al., 2001) that glia also increase the number of synapses that are form in cultured retinal ganglion cells and that glia are required for maintenance of the synapses that form.

The first paper of this dissertation investigated the effects of astrocytes on synapse formation in cultured hippocampal neurons. Finding from this study point to the fact that astrocytes greatly influence synaptogenesis but not in ways identical to what was found in the retinal system utilized by Barres. This study, therefore, opens the door to more questions. What are the specific astrocyte effects on local synaptogenesis throughout the nervous system? The second paper introduces a new imaging technique to perform biological near-field microscopy.

Dissertation Organization

This dissertation is composed of two papers. The papers are preceded by a general introduction that reviews the entire body of work and is followed by a general summary.

General references cited in the general introduction and general summary follow the appendices. The first paper (chapter 2), authored by R.T. Doyle, M. Mazzanti, A.A. Araque and P.G. Haydon will be submitted to the Journal of Neuroscience. I conceived the idea of astrocytes effects on synaptogenesis with Phil Haydon, designed the embryonic culture system that allowed us to produce astrocyte-deplete and astrocyte-enriched cultures that were essential for this work and performed all immunocytochemistry and electron microscopy for the study. Mary Mazzanti performed all the postnatal experiments and Alfonso Araque performed the electrophysiology of the embryonic cultures. The second paper (chapter 3), authored by R.T. Doyle, M. J. Szulzcewski and P.G. Haydon, has been accepted for publication by the Biophysical Journal. The majority of the effort for this paper was equally split between R.T. Doyle and P.G. Haydon.

There are three additional papers appended to the dissertation. Two of the papers represent investigations into synapse formation by identified neurons of the snail *Helisoma*. The remaining paper presents the effect of target contact on various aspects of synapse formation in cultured rat hippocampal neurons. My contribution to these papers is as follows: Appendix A, approximately 45%; Appendix B, approximately 40%; Appendix C, approximately 10%.

CHAPTER 2. ASTROCYTES SELECTIVELY ENHANCE HIPPOCAMPAL EXCITATORY SYNAPSE FORMATION AND AUGMENT THE MAGNITUDE OF THE N-TYPE CALCIUM CURRENT

A paper to be submitted to the Journal of Neuroscience

Robert T. Doyle, Mary Mazzanti, Alfonso Araque and Philip G. Haydon

ABSTRACT

The formation of the chemical synapse is a poorly understood process that is likely to critically rely on the presence of molecular cues that arise from presynaptic and postsynaptic neurons as well as associated glial cells. Since many synapses are wrapped by astrocytic processes we asked whether local interactions with these glial cells critically regulates synaptogenesis. Rat hippocampal cultures were established in astrocyte-deplete and enriched conditions in order to ask whether the presence of astrocytes stimulated the formation of the chemical synapse. Co-culture with astrocytes selectively augmented the formation of excitatory, but not inhibitory, chemical synapses, as detected by electrophysiological criteria. Additionally, immunostaining demonstrated that astrocytes stimulate the export of synaptic proteins from the cell body to the neurites, and presumably the nerve terminal, which on ultrastructural examination were shown to contain an increase in number of synaptic vesicles within the presynaptic terminal. In addition to actions on vesicles and vesicle proteins an analysis of the macroscopic calcium currents of hippocampal neurons revealed that local contact with astrocytes led to a selective augmentation of the magnitude of the N-type calcium current. Since the N-type calcium current is known to be associated with newly

formed synapses, we propose that local interactions between the process of an astrocyte and developing pre- and postsynaptic terminals will augment calcium influx and thus synaptic transmission to place this tripartite synaptic structure at a competitive advantage over neighboring developing synapses that are devoid of interactions with astrocytes.

INTRODUCTION

Glial cells fulfill many roles in the developing and mature nervous system. In addition to regulating neuronal migration, astrocytes, a sub-type of glial cell, support neuronal survival (Banker, 1980; Pfrieger and Barres, 1995; Raff et al., 1993; Ramon y Cajal, 1911; Tsacopoulos and Magistretti, 1996) and differentiation (Takeshima et al., 1994), neuronal guidance (Bastiani and Goodman, 1986; Kuwada, 1986; Rakic, 1990), neurite outgrowth (e.g., (Johnson et al., 1989; Le Roux and Reh, 1994; Noble et al., 1984; Smith et al., 1990), and control the local extracellular concentration of ions (Largo et al., 1996b; Orkand et al., 1966) and neurotransmitters (Kimelberg et al., 1990; Largo et al., 1996a; Mennerick and Zorumski, 1994; Szatkowski et al., 1990). During the past several years it has also been shown that after synapses form, neurons and astrocytes utilize chemical transmitters for bi-directional signaling (Attwell, 1994; Parpura et al., 1994; Pasti et al., 1997). For example, chemical transmitters released from synapses can elevate astrocytic calcium levels (Porter and McCarthy, 1996) which in turn cause the release of glutamate from this non-neuronal cell to modulate synaptic transmission (Araque et al., 1998a; Araque et al., 1998b; Araque et al., 1999b; Bezzi et al., 1998; Kang et al., 1998; Parpura et al., 1994). Thus, a picture is emerging in which astrocytes, and glial cells in general, are intimately involved in the regulation of nervous system development and function.

During the development of synaptic connections glial cells have been shown to regulate many neuronal properties including dendritic development, the expression of ligand- and voltage-gated ion channels, as well as the formation of functional synapses (Chamak et al., 1987; Chamak and Prochiantz, 1989; Denis-Donini et al., 1983; Denis-Donini et al., 1984; Li et al., 1999; Liu et al., 1997; Liu et al., 1996; Nakanishi et al., 1994; Pfrieger and Barres, 1997; Rouget et al., 1993; Rousselet et al., 1988; Ullian et al., 2001; Wu and Barish, 1994). The factors responsible for these developmental roles are not clearly established, although a complex interaction between vasoactive intestinal polypeptide, activity-dependent neurotrophic factor and neurotrophin 3 have been identified as necessary for synapse formation in cell culture (Blondel et al., 2000). Macroglial but not microglial cells increase the number and the strength of synapses formed by rat retinal ganglion cells in culture (Pfrieger and Barres, 1997) and secreted factors are required for the maintenance of synaptic connections (Ullian et al., 2001). Despite the importance of understanding the regulation of synapse formation, little knowledge is available for neurons isolated from the CNS.

The overall goal of this study was to determine whether astrocytes, the predominant form of glial cell of the central nervous system, regulate synaptogenesis of hippocampal neurons. In addition to studying the effects of astrocytes on the end-point, the formation of the synapse, we have also focused our study to ask whether astrocytes impact the appearance of individual components of the synapse, i.e., postsynaptic receptors, the calcium-sensitive vesicle protein synaptotagmin I, and specific calcium currents. In addition to demonstrating that astrocytes regulate synapse formation, this study provides three novel observations: astrocytes augment excitatory but not inhibitory synapses formation, astrocytes selectively

increase the magnitude of the N-type calcium current, and this augmentation results from local contact between the neuron and the glial cell.

MATERIALS AND METHODS

Embryonic hippocampal cultures

All studies, except for those in which calcium currents were monitored were performed with cultures made from embryonic hippocampi. For embryonic cultures, hippocampi were dissected from E18 Sprague-Dawley rat embryos and incubated for 15 minutes at room temperature in Earl's balanced salt solution (EBSS; pH 7.35,GIBCO) containing trypsin (0.25%; Sigma). The tissue was washed in fresh EBSS and placed in EBSS containing trypsin inhibitor (1.0 %; Sigma) for 5 minutes. Hippocampi were rinsed and then mechanically dissociated by trituration with a glass pipette in modified Minimum Essential Medium (MMEM) (GIBCO) containing 10 % FBS, 20 mM L-glutamine, 10 mM Na^+-pyruvate, 14mM Na-bicarbonate, 100 IU/ml pennicilin and 100 mg/ml streptomycin. Cells were plated onto either 12 mm glass coverslips coated with poly-L-lysine (1 mg/ml; MW 10,000; Sigma), (astrocyte-deplete cultures) or 12 mm coverslips which contained a monolayer of purified cortical astrocytes, (astrocyte-enriched cultures). Cultures were maintained at 37 °C in a 5 % CO_2 / 95 % room air atmosphere. The media was replaced one hour after plating with serum substituted MMEM containing N2 (GIBCO). N2 serum MMEM does not support non-neuronal cells and thus produced cultures highly enriched for neurons.

Preparation of cortical astrocyte cultures

Primary astrocyte cultures were established from neocortices of 0-3 day post-natal Sprague-Dawley (Harlan) rat pups according to previously established methods (Furshpan et al., 1986). All macro and micro dissection was carried out in ice-cold Earl's balanced salt solution (EBSS). Once removed, cortices were enzymatically treated with papain (20 U/ml) at 37°C in a 5%CO_2 and 95% air atmosphere for one hour. The enzymatic reaction was stopped by the addition of trypsin inhibitor (10 mg/ml). Tissue was transferred to MMEM and dissociated by gentle trituration through a glass serological pipette. The resulting cell suspension was placed into culture flasks (25 cm^2, Falcon).

After 10 to 14 DIV a purified astrocyte culture was produced by exchanging the media with ice-cold media and firmly tapping flasks, the flasks were then placed in an orbital shaker (260 rpm at 37°C) for 1.5 hours. After ice-cold media exchange, flasks were returned to the orbital shaker for an additional 18 hours. The cultures were enzymatically treated with trypsin (0.1%), to detach the cells from the flask. The enzymatic reaction was stopped with addition of fresh MMEM. The resulting cell suspension was transferred to a centrifuge tube, and astrocytes pelleted by centrifugation at 750 rpm for 10 minutes at room temperature. The pellet was then resuspended, gently triturated, and plated onto 12 mm diameter poly-l-lysine coated glass coverslips. These astrocytes monolayers were used in both embryonic and postnatal experiments. Dissociated embryonic hippocampal cells were plated onto astrocytes after 1-3 days. Astrocytes support neuronal survival (Banker, 1980; Chuah et al., 1991; Engele et al., 1991; O'Malley et al., 1994; Wang and Cynader, 1999; Yuzaki et al., 1993), and therefore will result in different cell densities in the two different culture conditions that could affect synapse formation (Fletcher et al., 1994). Therefore, cell plating was adjusted so

that neuronal densities in the two culture conditions were equivalent at the stage analyzed (8 DIV).

Preparation of post-natal hippocampal neuronal-enriched cultures

Primary hippocampal cultures were established from 0-3 day post-natal Sprague-Dawley (Harlan) rat pups in a manner similar to that described above for preparation of cortical astrocyte cultures. Cultures were plated in MMEM. On the third day in culture (3 DIV), 5-fluoro-2'-deoxyuridine (FUDR, 5 μM) was added to the medium to suppress proliferation of non-neuronal cells. Cultures were fed every four days with fresh MMEM.

Preparation of post-natal astrocyte-enriched and astrocyte-delete primary cultures.

In order to study astrocyte-mediated effects on neuronal calcium currents, we used a method in which we could choose hippocampal neurons that differ in their degree of contact with astrocytes. In this method, purified astrocytes (as described above) are plated on only one half of a 12 mm diameter coverslip. This is accomplished by placing a small volume of the astrocyte cell suspension (~50 μL) on one side of the coverslip. Once cells have attached to the poly-L-lysine substrate (about 3 hours) the entire coverslip is covered with medium (MMEM). After 2 to 3 DIV, these astrocytes will form a continuous layer that is restricted to approximately one half of the coverslip. Next, hippocampal neurons are dissociated (see above) and plated over the entire coverslip. The result is a single coverslip which contains astrocyte-enriched and astrocyte-deplete conditions. This method allows isolated neurons to be studied in the presence or absence of astrocyte contact on the same coverslip.

Preparation of hippocampal micro-island cultures

Coverslips containing hippocampal neurons growing on separated 'islands' of cortical astrocytes were established according to previously established methods (Furshpan et al.,

1986). These 'micro-island' cultures allowed control over neuron-neuron contact, since an island containing a single neuron, or an island containing a pair of neurons could be chosen for study.

Immunocytochemistry.

Localization of the synaptic vesicle protein synaptotagmin I and MAP2, a neuron-specific microtubule-associated protein that is restricted to the somatodendritic area of neurons (Caceres et al., 1986), was accomplished by indirect immunocytochemistry. The primary monoclonal antibody directed against synaptotagmin I (Cl 41.1) was kindly provided by Dr. R. Jahn. Monoclonal antibody against MAP2 was purchased from Sigma Chemical Company. Both antibodies were used at a 1:250 dilution in a phosphate buffered saline (PBS) solution containing 0.5% BSA, 0.5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3. Cultures were fixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes before being rinsed and permeabilized with Triton X-100 (0.25% in PBS). The tissue was then incubated in a PBS solution containing 5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3 for 30 minutes to block non-specific binding. Cultures were incubated with primary antibody overnight at 4 °C. Primary antibody was removed, cells washed and then incubated in rhodamine-conjugated goat anti-mouse secondary antibodies (1:200; Fisher Scientific International, Pittsburg, PA) for 2 hours. The percentage of inhibitory neurons in embryonic cultures was determined using glutamic acid decarboxylase (GAD) immunocytochemistry. The GAD6 antibody developed by Dr. D. I. Gottlieb (Chang and Gottlieb, 1988) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242 under contract NO1-HD-7-3263 from NICHD.

Electron Microscopy.

Embryonic cultures were fixed at 8 days in vitro (DIV) with 1.25 % glutaraldehyde in 0.1M sodium cacodylate for 30 minutes at room temperature. After rinsing in sodium cacodylate, they were post-fixed with 0.67 % OsO_4 in 0.1M sodium cacodylate for 30 minutes. The cultures were first rinsed with sodium cacodylate and then sodium acetate (0.1M) before being en bloc stained with 1% uranyl acetate for 45 minutes in the dark. The tissue was dehydrated in an increasing series of ethanol with three changes of 100% ethanol. Ethanol was replaced by propylene oxide before infiltration with an increasing concentration of EPON resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 9 electron microscope.

Electrophysiology.

Whole-cell patch clamp recordings were obtained from neurons with an Axopatch-1C amplifier. The external solution contained (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, 6 Sucrose, pH 7.35. Patch pipettes had d.c. resistances of 5-10 MΩ when filled with internal solution that contained (in mM): 140 potassium gluconate, 10 EGTA, 4 Mg-ATP, 0.2 Tris-GTP, 10 HEPES, pH 7.35. The membrane potential was usually held at -70 mV, and varied up to -35 mV to test for the presence of inhibitory postsynaptic currents. The presence of spontaneous postsynaptic currents (sPSCs) was assayed for a three-minute period. Subsequently, up to 3 potential presynaptic neurons that clearly appeared to make physical contact with a potential postsynaptic neuron, were extracellularly stimulated by applying voltage pulses (0.3-1 ms duration; 60-150 V; delivered at s^{-1}) between a platinum reference electrode and a external solution-filled micropipette (5-10 MΩ d.c. resistance) placed over the neuronal soma. Ten to 50 evoked postsynaptic currents were recorded.

Currents were filtered at 1-2 kHz, sampled above 1 kHz and acquired using pClamp software (Axon Instruments, Foster City, CA). Analysis of sPSCs was done using the ACSPLOUF software (obtained from Dr. Pierre Vincent, University of California at San Diego). The presence of glutamate receptors was tested by using brief (< 50 ms) pressure-mediated (Picospritzer II, General Valve, Fairfield, NJ) ejections of glutamate (0.5 mM; sodium salt in external solution) from a micropipette placed over the neuronal soma. All recordings were performed at room temperature (20-22 °C). Data obtained from at least 3 different cultures are expressed as mean ± s.e.m., and statistical differences were established using the Student's t-test, unless otherwise indicated. At least 6 postsynaptic and 19 potential presynaptic neurons were recorded and stimulated, respectively, in each culture condition and per experiment.

To record macroscopic calcium currents, patch pipettes were filled with (in mM): 28 TEA, 14 di-sodium phosphocreatine, 10 EGTA, 10 HEPES, 2 $MgCl_2$, 4 MgATP, 0.3 TrisGTP, and 0.1 Leupeptin. The external bath solution contained (in mM): 100 NaCl, 10 TEA-Cl, 2 $CaCl_2$, 5 CsCl, 2 $MgCl_2$, 10 Glucose, 10 HEPES, and 0.001 TTX. Pipette and bath solutions were adjusted to 300 and 315 mOsmoles, respectively, and both solutions were adjusted to 7.35 pH.

Whole-cell calcium currents were evoked by depolarizing steps from a negative holding potential. Five fractionally scaled hyperpolarizing sub-pulses were used to subtract leakage current on-line. Total inward current (pA) evoked by the depolarizing step was divided by the whole-cell membrane capacitance (pF), to obtain a measure of whole-cell current density (pA/pF). In addition to measures of current density at a single depolarizing potential, a complete current-voltage (I-V) relation was obtained in each cell by stepping (in

+10 mV increments) through a series of depolarizing voltages from a negative holding potential.

RESULTS

Astrocytes increase the formation of excitatory synapses between hippocampal neurons

To assess the impact of astrocytes on synapse formation we cultured neurons in astrocyte-deplete and astrocyte-enriched conditions for eight days and then assayed connectivity based on the formation of functional chemical connections as assessed by electrophysiological criteria. Irrespective of the culture condition, neurons were able to generate all-or-none action potentials (Figure 1A), and displayed spontaneous and evoked postsynaptic currents (PSCs, Figure 1B,C). Both excitatory and inhibitory synapses were able to form between cultured neurons. However, co-culture with astrocytes augmented the formation of excitatory connections. The probability that a neuron exhibited spontaneous excitatory postsynaptic currents (sEPSCs) increased from 17.14 ± 10.17 % in astrocyte-deplete cultures (n=31) to 61.1 ± 5.6 % in astrocyte-enriched cultures (n=23, $P < 0.05$). Similarly, the probability of detecting evoked excitatory synaptic transmission increased from 9.41 ± 3.4 % in astrocyte-deplete cultures (n=95) to 60.25 ± 13.0 % in astrocyte-enriched cultures (n=72, $P < 0.001$; Figure 1D). This increased detection of excitatory synapses was not the result of an action on cell density because after 8 DIV cultures showed 58.0 ± 2.3 (n = 370 fields, field = 700 x 450 μm) and 54.7 ± 1.6 (n= 330 fields) neurons per field in astrocyte-deplete or astrocyte-enriched conditions, respectively. While the proportion of excitatory connections detected in cultures was augmented, the mean amplitude and frequency of both excitatory and inhibitory

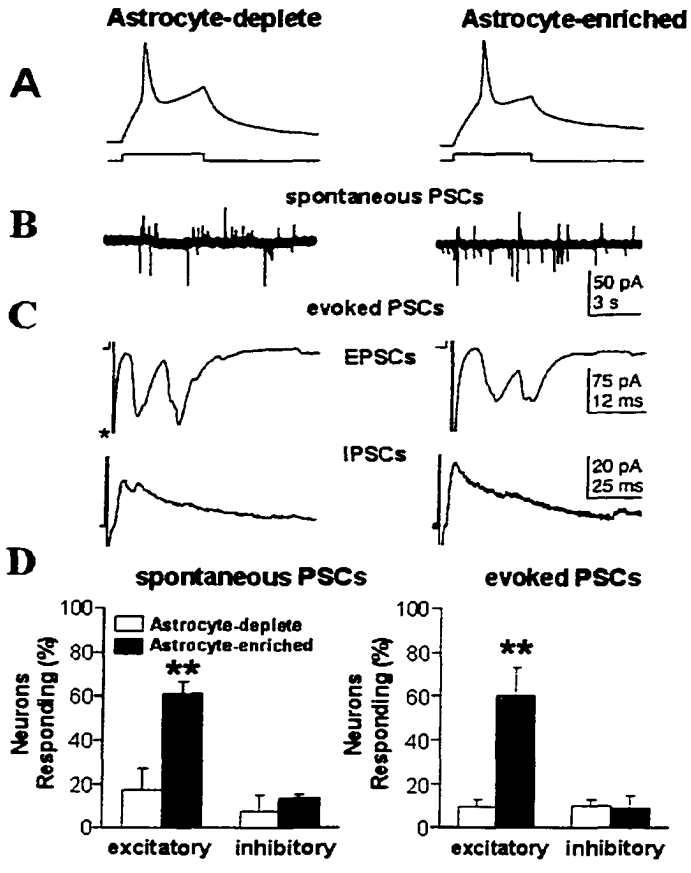

Figure 1. Astrocytes selectively stimulate the formation of excitatory chemical synapses between cultured hippocampal neurons. Electrophysiological properties of rat hippocampal neurons cultured in astrocyte-deplete or astrocyte-enriched conditions after 8 DIV. Neurons in both conditions exhibit regenerative action potentials (A), spontaneous (B) and evoked (C) synaptic transmission (asterisk denotes stimulus artifact). However, the percentage of neurons displaying either spontaneous (D) or evoked (E) excitatory synaptic currents is augmented by co-culture with astrocytes, while inhibitory responses are unaffected by co-culture with these glial cells.

synaptic currents were not found to be significantly different in the two conditions (data not shown).

In comparison to excitatory connections, co-culture with astrocytes did not affect the ability to detect functional inhibitory synapses. sIPSCs were recorded in equal proportion in

neurons cultured in either astrocyte-deplete (7.5 ± 7.5 %) or in astrocyte-enriched conditions (13.43 ± 1.67 %) and evoked IPSCs were similarly unchanged (10.03 ± 2.91 % astrocyte-deplete and 8.97 ± 5.58 astrocyte-enriched; Figure 1D). The differential ability of astrocytes to augment excitatory compared to inhibitory synapses is not due to a selective ability to regulate the number of glutamatargic and GABAergic neurons. Using immunolocalization of antibodies against microtubule-associated protein (MAP2; (Caceres et al., 1986; Craig et al., 1993) and the synthetic enzyme glutamic acid decarbolxylase (GAD, a selective marker for inhibitory neurons) we found that the proportion of inhibitory neurons in both culture conditions was not significantly different (9.45 ± 0.99 % and 8.08 ± 0.42 in astrocyte-deplete and astrocyte-enriched cultures, respectively.) Since neurons normally receive inhibitory as well as excitatory synaptic inputs, the inability of astrocytes to increase the proportion of inhibitory synapses in our cultures suggests that inhibitory and excitatory synapse formation are subject to differential regulation.

To ask whether the enhancement of synapse formation detected in the presence of astrocytes resulted from a change in presence of functional glutamate receptors we pressure ejected glutamate onto neurons and determined whether they were sensitive to this neurotransmitter. Whereas the proportion of neurons exhibiting evoked excitatory connections was augmented by the presence of astrocytes, these non-neuronal cells did not regulate whether neurons were sensitive to glutamate. Pressure ejection of glutamate onto the somata of neurons evoked glutamate-dependent inward currents in all neurons tested (n = 14) that were co-cultured with astrocytes, and in 81% of neurons (n = 37) that were cultured in astrocyte-deplete conditions. Thus, the proportion of neurons with functional glutamate

receptors, and the amplitude of synaptic currents, an indirect assay of sensitivity to glutamate, were unaffected by co-culture with astrocytes.

Astrocytes stimulate both pre and postsynaptic neuronal maturation

As a second test of the ability of astrocytes to regulate synapse formation we examined the ultrastructure of neuronal processes. Examination of synapses by transmission electron microscopy corroborate our conclusion that astrocytes enhance synapse formation because astrocytes increased the number of synapses in cultures, and stimulated the maturation of presynaptic terminals. To determine the relative number of synapses we developed a relative quantification approach in which we determined the probability of detecting multiple presynaptic terminals in the same area of examination. Putative presynaptic terminals were identified based on the presence of closely apposed membranes with typical pre- and postsynaptic densities and synaptic vesicles in the presynaptic zone (Figure 2A) (cf.(Pfrieger and Barres, 1997)). To estimate the number of synapses, one synapse was placed in the center of the image area (96 x 112 μm) and then the number of additional synapses present in that area of examination were counted. Co-culture with astrocytes increased the number of synapses two-fold. On average, 1.15 ± 0.16 additional synapses were found in neurons co-cultured with astrocytes, while only 0.55 ± 0.13 additional synapses were found in astrocyte-depleted cultures.

While this observation clearly supports an augmentation of synapse formation in general, our ultrastructural investigation also demonstrated that the presence of astrocytes enhanced the maturation of individual presynaptic terminals. In the presence of astrocytes, presynaptic terminals had a greater number of synaptic vesicles (40.9 ± 3.2, n = 136 compared with 23.6 ± 1.9, n = 93; Figure 2), and an increase in the number of vesicles found

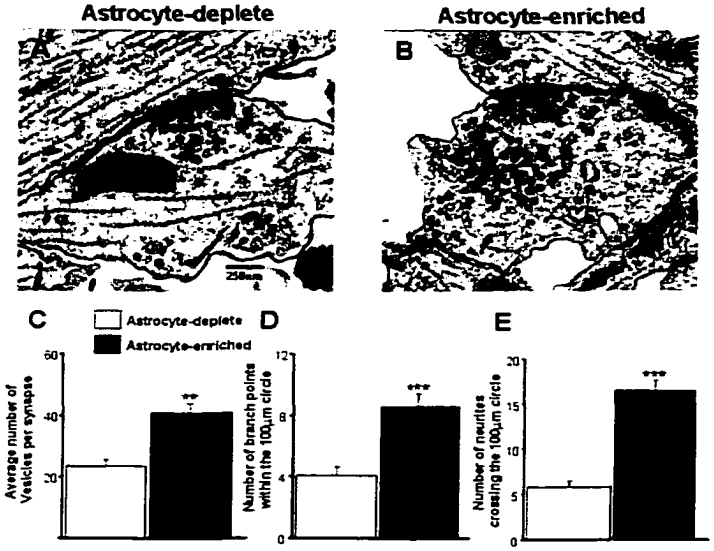

Figure 2. Astrocytes stimulate both pre- and post-synaptic maturation. (A) and (B) show typical examples of synaptic terminals that were identified with transmission electron microscopy in astrocyte-deplete and enriched conditions. A quantitative analysis of synapses revealed that there were a greater number of vesicles per synapse when neurons were cultured in astrocyte-enriched conditions (C). Immunostaining of cultures with anti-MAP2 to disclose the location of neuronal dendrites demonstrated that neurons cultured in astrocyte enriched conditions exhibited (D) a greater degree of dendritic branching and (E) a greater number of dendrites compared to neurons cultured in astrocyte-deplete conditions

within one vesicle diameter of the presynaptic plasma membrane (3.0 ± 0.17, n = 136 compared with 1.9 ± 0.17, n = 93) which we call morphologically docked vesicles.

Several studies have shown that astrocytes support neurite outgrowth (e.g., (Johnson et al., 1989; Le Roux and Reh, 1994; Noble et al., 1984; Smith et al., 1990). Therefore, astrocytes could increase the number of synapses by increasing the effective area of possible contact between axons and dendrites. To analyze the possible morphological differences in the dendrites of neurons grown in the presence and the absence of astrocytes after 8 DIV we mapped the dendritic arbors using an antibody against MAP2 (Caceres et al., 1986; Craig et al., 1993). In order to quantify the extent and complexity of the dendrites, two parameters

were measured. We centered a 100μm circle over images of neuronal cell bodies and determined the number of dendritic branch points within this area, as well as the number of MAP2 positive processes that crossed the circle. Astrocyte-deplete cultures averaged 4.1 ± 0.58 branch points within the circle and 5.9 ± 0.72 processes crossing the circle (n=28, Figure 2D, E) while astrocyte-enriched cultures displayed 8.6 ± 0.81 branch points in the circle and 16.5 processes crossing the circle (n=21, Figure 2D, E). The presence of astrocytes resulted in a significant increase in dendritic development ($p<0.001$) and therefore increased possibilities of axon and dendrite interaction.

Some presynaptic proteins have been shown to undergo developmental regulation that is temporally correlated with the establishment of functional synapses (Basarsky et al., 1994; Benson and Cohen, 1996; Fletcher et al., 1991; Matteoli et al., 1991; Rene et al., 1997). In cultured hippocampal neurons the distribution of the synaptic vesicle protein synaptotagmin I, changes with synaptic maturation. At early stages, synaptotagmin immunoreactivity is primarily localized in neuronal cell bodies, while neurons at mature stages display intense punctate staining along processes and with an accompanying loss of signal in the soma (Figure 3) (Basarsky et al., 1994). Using an antibody against the presynaptic calcium-sensitive vesicle protein, synaptotagmin I, we asked whether the presence of astrocytes augmented presynaptic maturation by determining the proportion of neurons with anti-synaptotagmin immunoreactivity in the neuronal cell body. Synaptotagmin immunoreactivity was diffusely distributed within neurons at early stages in culture (1-3 DIV) with accumulations in the cell bodies (Figure 3A,B, arrows,). Further development was marked by a loss of cell body staining and an increase in punctate staining in neurites (8 DIV, 4C). We asked whether this developmental pattern was influenced by astrocytes, by determining the

proportion of neurons exhibiting synaptotagmin I immunoreactivity in their cell body in the two culture conditions. Significant differences between the two culture conditions were seen after 3 DIV (Figure 3D, $p < 0.001$) and were more prominent at 8 DIV. This result clearly demonstrates that astrocytes accelerate the timing of when synaptotagmin I is exported from the cell body to presynaptic sites and support the hypothesis that astrocytes regulate presynaptic maturation.

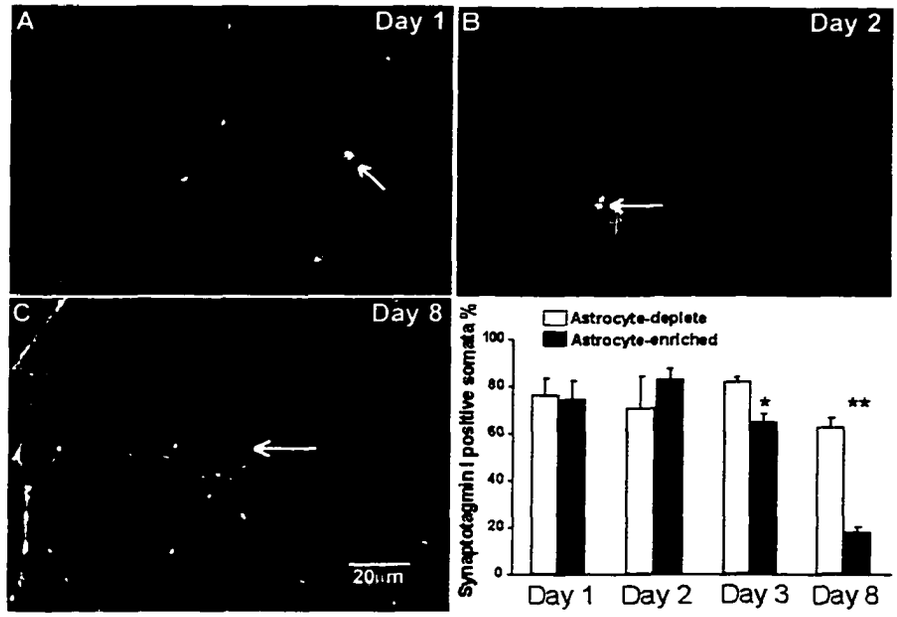

Figure 3. Astrocytes stimulate the export of the synaptic protein synaptotagmin I from the neuronal cell body. Immunostaining for synaptotagmin I was performed on hippocampal neurons cultured in astrocyte-deplete and astrocyte-enriched conditions (A-C). After 1 day in culture synaptotagmin I is predominately located in the neuronal cell body but with time in culture this synaptic protein can be seen to accumulate in a punctate pattern in neuronal processes (B, C). After eight days in culture in the presence of astrocytes, neurons are largely devoid of peri-nuclear accumulations of synaptotagmin (arrow) and the majority of the immunostaining is localized within the neuronal processes (C). Neurons were cultured in astrocyte-deplete and enriched conditions. A significant difference in synaptotagmin I distribution between neurons in the two culture conditions was seen by day 3 (D) that continued to increase until day 8, demonstrating that astrocytes stimulate the export of the synaptic protein synaptotagmin I from the neuronal cell body to neuronal processes.

Contact between astrocytes and neurons selectively augments the magnitude of the N-type calcium current

Calcium influx through specific voltage-sensitive calcium channels is essential to stimulate neurotransmitter release. How these calcium channels, and specifically the current essential for transmitter release, are regulated during synapse formation is largely undefined. While neuron-neuron contact can influence the distribution of calcium channels (Bahls et al., 1998), and co-culture with macroglia can augment calcium currents (Ullian et al., 2001) whether astrocytes also impart developmental regulation is poorly understood.

Macroscopic calcium currents were measured in whole-cell voltage clamp recordings from hippocampal somata (1-2 DIV) under conditions in which hippocampal neurons were either in contact with, or isolated from, astrocytes. Neurons in astrocyte-enriched conditions displayed significantly larger macroscopic calcium currents than neurons in astrocyte-deplete conditions (Figure 4, $n=8$, $p<0.01$) with no shift in voltage-activation threshold between the two groups. What is more, the astrocyte-induced augmentation of the calcium current is a transient phenomenon since it is only detected at early times in culture. The astrocyte-induced enhancement of calcium current density was much greater in young (1 to 3 DIV, 435%) as compared to mature (7, 8, and 9 DIV, 2%) neurons.

Astrocytes enhance the magnitude specifically of the N-type calcium current. Whole-cell calcium currents were measured in voltage clamp recordings before and after the addition of one of three calcium channel antagonists, each acting specifically to block a sub-type of calcium channel as follows; Nifedipine (10 μM) for L-type; ω-CgTX GVIA (1 μM) for N-type; and ω-Aga-IVA (500 nM) for P/Q-type. Drugs were pressure ejected from a glass microcapillary tube with a tip opening diameter of 3 to 5 μm. N-type calcium current

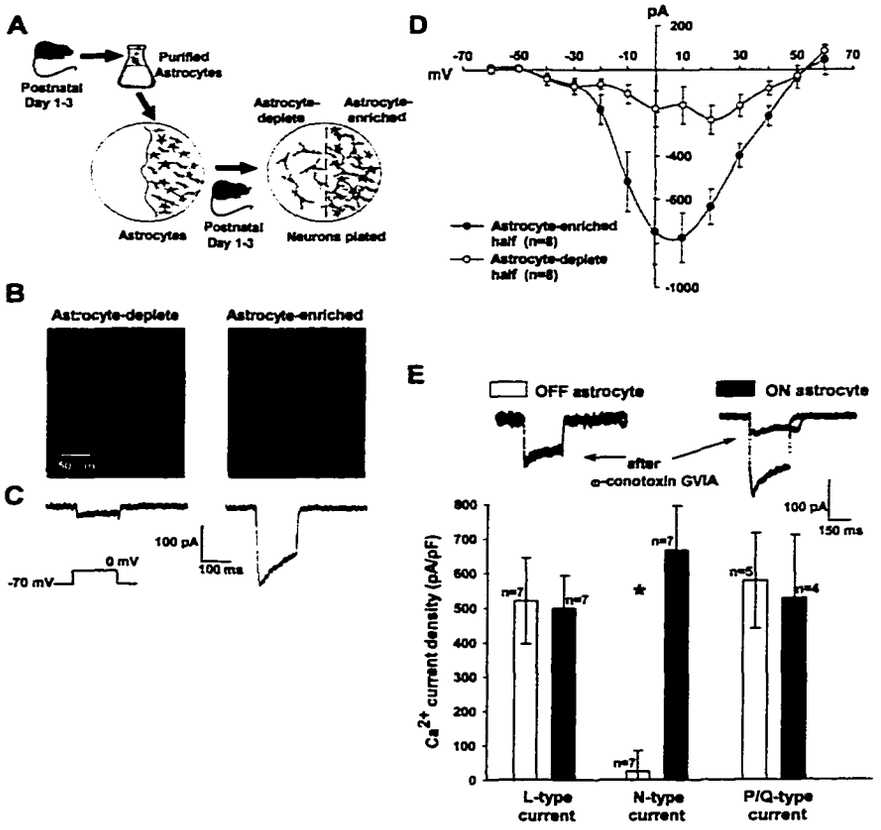

Figure 4. Astrocytes stimulate the appearance of functional N-type calcium currents in hippocampal neurons. (A) Astrocyte-deplete and astrocyte-enriched neuronal cultures were established by pre-plating purified astrocytes on one-half of a coverslip followed by a secondary plating of hippocampal neurons. (B) Phase-contrast images showing neurons being recorded in astrocyte-deplete and enriched conditions with representative traces (3 DIV) shown in (C). Neurons in the astrocyte-enriched condition (n=9) showed significantly larger calcium currents than neurons in the astrocyte-deplete condition (n=9) ($p < 0.05$, student's t-test). Each trace represents the average of 4 individual traces. (D) Current-voltage (i/v) relation of neuronal calcium currents showing similar i/v relation in astrocyte-deplete and enriched conditions. (E) Demonstrates that astrocytes selectively enhance the magnitude of the N-type calcium current. (Top) Representative traces showing the reduction of calcium current caused by the application of the N-type calcium channel antagonist ω-conotoxin GVIA (1 μM). (Bottom) Pharmacological dissection of the calcium current using nifedipine (10μM), ω-conotoxin GVIA (1 μM) and ω-agatoxin IVA (500nM) to identify the contribution of the L-type, N-type and P/Q type calcium currents to the whole-cell calcium current of neurons cultured on and off astrocytes. Note that the enhancement of calcium current seen in astrocyte-enriched conditions is due to the increase in the N-type calcium current ($p < 0.01$, ANOVA).

was significantly enhanced in neurons contacting astrocytes (Figure 4E) while L-type and P/Q-type currents were unchanged. The selective action of astrocytes on the N-type calcium current is particularly exciting because this current is the predominant type that is linked to transmitter release in developing synapses (Rosato Siri and Uchitel, 1999; Scholz and Miller, 1995; Verderio et al., 1995).

Since astrocytes augment neurite extension (Fallon, 1985; Goodman et al., 1993; Le Roux and Reh, 1994; van den Pol and Spencer, 2000; Wang et al., 1994) and because neuron-neuron contact is known to affect the distribution of the N-type calcium channel (Bahls et al., 1998), it is important to determine whether the astrocyte-induced augmentation of the N-type current is secondary to effects mediated by neuron-neuron contact. In order to control for this possibility neurons were plated in microisland culture conditions where we could select either single or paired neurons on astrocyte microislands (Figure 5). We detected no difference in the magnitude of the macroscopic calcium current in single or paired neurons suggesting that astrocytes directly regulate the appearance of the N-type calcium current.

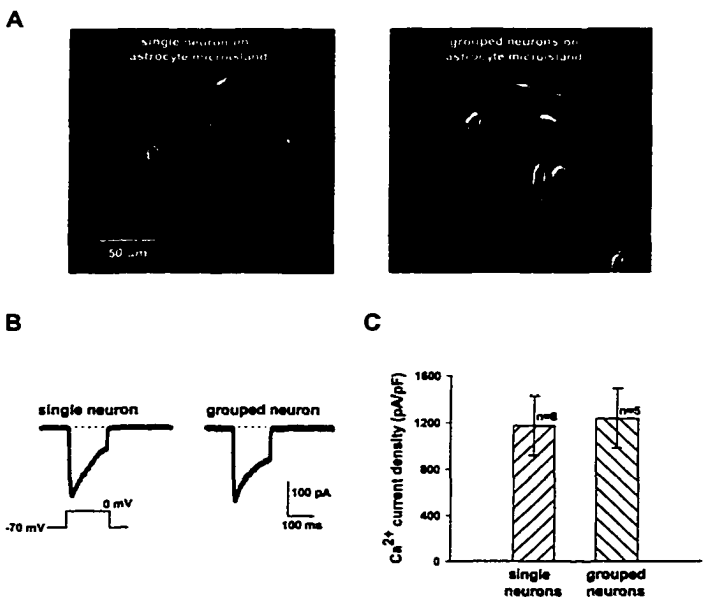

Figure 5. Neuron-neuron interactions do not increase the calcium current density. (A) Phase-contrast images showing single (left) and multiple (right) neurons on astrocyte micro-islands. (B) Representative traces recorded from a solitary neuron (left) and grouped (right) neurons after 3 days in culture on astrocyte micro-islands. Each trace represents the average of 4 individual records. Currents were evoked as described in Fig 4(C). (C) summarizes calcium current densities recorded from grouped (n=6) and solitary (n=6) neurons demonstrating that neuron-neuron contact does not augment calcium current density.

Astrocytes are known to be the source of many signaling molecules (such as growth factors) that can act at a distance to affect the physiology of neurons. Indeed glial-conditioned medium has been shown to regulate synapse formation (Pfrieger et al, 1997; Ullian et al, 2001; Blondel et al, 2000). In order to assess the role of contact in the astrocyte-induced enhancement of the N-type calcium current, we plated neurons into relatively low density conditions so that some would make contact with an astrocyte (ON), while others would be isolated (OFF). We then performed a paired analysis in which calcium current magnitudes were monitored from each of two adjacent neurons, one in the 'on-astrocyte' and the other in the 'off-astrocyte' configuration (Figure 6). Neuronal cell bodies that contact an astrocyte displayed significantly larger calcium currents than neurons in the off-astrocyte configuration ($p<0.01$). Since it is likely that neurons in each condition are exposed to similar secreted factors, our results indicate that contact between an astrocyte and neuron is required for the augmentation of the calcium current.

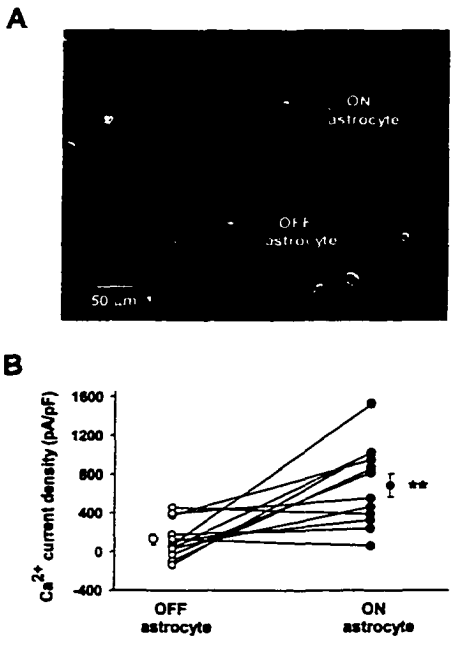

Figure 6. Astrocyte contact is necessary for enhancement of calcium current. (A) Phase-contrast image showing a neuronal cell body in contact with an underlying astrocyte (ON astrocyte) and another nearby neuron in which the cell soma does not contact an astrocyte (OFF astrocyte). (B) The Ca^{2+} current density was recorded from pairs of neurons in ON (n=12) and OFF (n=12) conditions. Group means are represented by the single filled circles. Neurons in the ON-astrocyte condition have significantly greater magnitude Ca^{2+} current density than neurons in the OFF-astrocyte condition ($p<0.01$, paired t-test).

DISCUSSION

The results of this study demonstrate that astrocytes stimulate the formation of excitatory synapses between cultured hippocampal neurons. Significantly more excitatory synapses form in astrocyte-enriched conditions as assessed by the export of the vesicle protein synaptotagmin I from the neuronal cell body to the processes, the number of vesicles within synaptic terminals, the magnitude of the calcium current, as well as the incidence of functional synaptic transmission. These data are in accordance with other recent studies that collectively demonstrate a critical role for glia in the regulation of synaptogenesis. For example, diffusible factors released from macroglia, but not microglia, stimulate the formation of synapses by retinal ganglion cells. Blondel et al. (Blondel et al., 2000) have shown that the release of activity-dependent neurotrophic factor from hippocampal astrocytes stimulates hipocampal synaptogenesis in culture. In support of a role for glia in the regulation of synaptogenesis Ullian et al (2001) recently demonstrated a coincidence in timing of the appearance of immunoreactivity for the vesicle protein SV2 and the appearance of S100β-positive astrocytes in the superior colliculus. Thus, not only do astrocytes stimulate synapse formation *in vitro*, but this non-neuronal cell type appears in the nervous system at a time that temporally coincides with synaptogenesis *in vivo*.

While there are great similarities between this study and others that have been recently reported, we also find subtle differences. For example, studies of the synaptogenesis by retinal ganglion cells show an increase in the frequency and magnitude of synaptic events, and ultrastructural studies did not detect a difference in the number of vesicles within individual synapses (Pfrieger and Barres, 1997). It is possible that these differences arise as a result of regional cell-specific differences, or due to the effects of diffusible factors versus

local contact. Since we found that astrocytes selectively augment excitatory but not inhibitory synaptogenesis, it is likely that glial cells do exert different effects on distinct classes of neuron.

While chemical factors released from glial cells do impact synaptogenesis, we provide direct support for the possibility that local contact between the astrocyte and the developing neurons impacts specific aspects of presynaptic development. We demonstrate that contact between an astrocyte and a neuron dramatically and selectively upregulates the magnitude of the N-type calcium current. That only the N-type calcium current, and that neither the L- nor P/Q-type currents were affected is in accordance with previous observations that the N-type current predominately supports synaptic transmission at developing synapses (Chameau et al., 1999; Iwasaki et al., 2000; Iwasaki and Takahashi, 1998; Scholz and Miller, 1995; Scholz and Miller, 1996). Whether contact with an astrocyte regulates only those N-type calcium channels in the local contact vicinity, or whether it causes a global 'switch-like' activation of channels throughout the whole neuron is likely to be an important issue that may impact the spatial specificity of synaptogenesis. Perhaps chemical factors released from astrocytes 'prime' the neuron so that it is prepared for synaptogenesis, and then local physical interactions regulate spatially discrete aspects of synaptogenesis.

In the mature nervous system many synapses consist of three structures, the presynaptic and the postsynaptic terminals that are wrapped by the process of an astrocyte (Araque et al., 1999a). What mechanism might control this tripartite spatial association? It seems unreasonable to assume that this structure arises through only random interactions. Perhaps the chance local interaction of a developing synapse with the process of an astrocyte

will put that local connection at a competitive advantage over neighbors so that the tripartite structure is stabilized. Certainly there is abundant evidence supporting the possibility that competitive interactions can affect the outcome of synaptogenesis (Bodnarenko and Chalupa, 1993; Katz and Constantine-Paton, 1988; Kossel et al., 1995; Shatz, 1990; Wong et al., 1991). Whether astrocytes can influence synaptic competition is largely undefined. However, transplantation of astrocytes into the adult cat visual cortex does permit ocular dominance plasticity, a process that requires synaptic competition (Muller and Best, 1989). Perhaps the local interaction between an astrocytic process and the developing presynaptic terminal causes the appearance of N-type calcium channels and the formation of a functional nerve terminal in which action potentials can release neurotransmitter. In such a situation, the developing tripartite synapse could be at a competitive advantage over neighboring synapses, which did not make contact with an astrocyte, and thus would be stabilized.

The past decade has seen an exciting increase in our understanding of glial cell biology (Araque et al., 2001; Araque et al., 1999b; Barres, 1991a; Barres, 1991b; Barres, 1991c; Barres et al., 1990; Carmignoto, 2000; Charles, 1994; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Haydon, 2001; Smith, 1992; Vesce et al., 1999). While once thought to be merely supportive cells, glia have been demonstrated to be involved in a reciprocal signaling pathway with neurons: neuronal activity can cause transmitter-induced calcium signaling in glia, which leads to the feedback release of chemical transmitters from the glial cells. (Araque et al., 2001; Araque et al., 1998a; Araque et al., 1998b; Araque et al., 1999b; Cornell-Bell et al., 1990; Dani and Smith, 1995; Haydon, 2001; Nedergaard, 1994; Parpura et al., 1994; Pasti et al., 1997; Porter and McCarthy, 1996; Sanzgiri et al., 1999). How this signaling regulates information processing is not yet defined, but it is clear that

chemical transmitters that are released from glial cells can modulate neighboring synapses. (Araque et al., 1998a; Araque et al., 1998b; Araque et al., 1999b; Castonguay and Robitaille, 2001; Robitaille, 1998). Reciprocal signaling between neurons and glia also extends to the developing nervous system. Astrocytes regulate synapse formation (Doyle et al., 1996; Nakanishi et al., 1994; Pfrieger and A., 1996; Pfrieger and Barres, 1997; Ullian et al., 2001), and the ensuing synaptic activity regulates calcium signaling between astrocytes (Haydon, 2000; Rouach et al., 2000).

In conclusion, this study adds to a growing body of literature that demonstrates that astrocytes, and macroglia in general, significantly influence the formation of the synapse. While neurons are competent to form synapses, diffusible factors can upregulate the number and strength of synapses that do form. Astrocytes exert influences at many levels by stimulating dendritic development, the export of the vesicle protein synaptotagmin I, an enhanced accumulation of presynaptic vesicles. In this study we also directly demonstrate that contact between an astrocyte and a neuron selectively augments the N-type calcium current, which is known to be essential for synaptic transmission. We propose that local contact between an astrocyte and a presynaptic terminal will enhance local synaptic transmission at that terminal, putting it at a competitive advantage over other developing, astrocyte-devoid synapses.

REFERENCES

Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci.* 10:2129-42.

Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1999a. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22:208-15.

Araque, A., R.P. Sanzgiri, V. Parpura, and P.G. Haydon. 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci.* 18:6822-9.

Araque, A., R.P. Sanzgiri, V. Parpura, and P.G. Haydon. 1999b. Astrocyte-induced modulation of synaptic transmission. *Can J Physiol Pharmacol.* 77:699-706.

Attwell, D. 1994. Glia and neurons in dialogue. *Nature.* 369:707-8.

Bahls, F.H., R. Lartius, L.E. Trudeau, R.T. Doyle, Y. Fang, D. Witcher, K. Campbell, and P.G. Haydon. 1998. Contact-dependent regulation of N-type calcium channel subunits during synaptogenesis. *J Neurobiol.* 35:198-208.

Banker, G.A. 1980. Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science.* 209:809-10.

Basarsky, T.A., V. Parpura, and P.G. Haydon. 1994. Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. *J Neurosci.* 14:6402-11.

Bastiani, M.J., and C.S. Goodman. 1986. Guidance of neuronal growth cones in the grasshopper embryo. III. Recognition of specific glial pathways. *J Neurosci.* 6:3542-51.

Benson, D.L., and P.A. Cohen. 1996. Activity-independent segregation of excitatory and inhibitory synaptic terminals in cultured hippocampal neurons. *J Neurosci.* 16:6424-32.

Bezzi, P., G. Carmignoto, L. Pasti, S. Vesce, D. Rossi, B.L. Rizzini, T. Pozzan, and A. Volterra. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature*. 391:281-5.

Blondel, O., C. Collin, W.J. McCarran, S. Zhu, R. Zamostiano, I. Gozes, D.E. Brenneman, and R.D. McKay. 2000. A glia-derived signal regulating neuronal differentiation. *J Neurosci*. 20:8012-20.

Bodnarenko, S.R., and L.M. Chalupa. 1993. Stratification of ON and OFF ganglion cell dendrites depends on glutamate-mediated afferent activity in the developing retina. *Nature*. 364:144-6.

Caceres, A., G.A. Banker, and L. Binder. 1986. Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. *J Neurosci*. 6:714-22.

Castonguay, A., and R. Robitaille. 2001. Differential Regulation of Transmitter Release by Presynaptic and Glial Ca2+ Internal Stores at the Neuromuscular Synapse. *J Neurosci*. 21:1911-22.

Chamak, B., A. Fellous, J. Glowinski, and A. Prochiantz. 1987. MAP2 expression and neuritic outgrowth and branching are coregulated through region-specific neuro-astroglial interactions. *J Neurosci*. 7:3163-70.

Chamak, B., and A. Prochiantz. 1989. Influence of extracellular matrix proteins on the expression of neuronal polarity. *Development*. 106:483-91.

Chameau, P., P. Lucas, K. Melliti, R. Bournaud, and T. Shimahara. 1999. Development of multiple calcium channel types in cultured mouse hippocampal neurons. *Neuroscience*. 90:383-8.

Chang, Y.C., and D.I. Gottlieb. 1988. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci.* 8:2123-30.

Chuah, M.I., S. David, and O. Blaschuk. 1991. Differentiation and survival of rat olfactory epithelial neurons in dissociated cell culture. *Brain Res Dev Brain Res.* 60:123-32.

Craig, A.M., C.D. Blackstone, R.L. Huganir, and G. Banker. 1993. The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron.* 10:1055-68.

Denis-Donini, S., J. Glowinski, and A. Prochiantz. 1983. Specific influence of striatal target neurons on the in vitro outgrowth of mesencephalic dopaminergic neurites: a morphological quantitative study. *J Neurosci.* 3:2292-9.

Denis-Donini, S., J. Glowinski, and A. Prochiantz. 1984. Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurones. *Nature.* 307:641-3.

Doyle, R.T., A.A. Araque, and P.G. Haydon. 1996. Astrocytes enhance synapse formation between cultured rat hippocampal neurons. Abstract, Society for Neuroscience. Vol. 1996. 1949.

Engele, J., D. Schubert, and M.C. Bohn. 1991. Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: role of mesencephalic glia. *J Neurosci Res.* 30:359-71.

Fallon, J.R. 1985. Neurite guidance by non-neuronal cells in culture: preferential outgrowth of peripheral neurites on glial as compared to nonglial cell surfaces. *J Neurosci.* 5:3169-77.

Fletcher, T.L., P. Cameron, P. De Camilli, and G. Banker. 1991. The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J Neurosci.* 11:1617-26.

Furshpan, E.J., S.C. Landis, S.G. Matsumoto, and D.D. Potter. 1986. Synaptic functions in rat sympathetic neurons in microcultures. I. Secretion of norepinephrine and acetylcholine. *J Neurosci.* 6:1061-79.

Goodman, M.N., J. Silver, and J.W. Jacobberger. 1993. Establishment and neurite outgrowth properties of neonatal and adult rat olfactory bulb glial cell lines. *Brain Res.* 619:199-213.

Haydon, P.G. 2000. Neuroglial networks: neurons and glia talk to each other. *Curr Biol.* 10:R712-4.

Iwasaki, S., A. Momiyama, O.D. Uchitel, and T. Takahashi. 2000. Developmental changes in calcium channel types mediating central synaptic transmission. *J Neurosci.* 20:59-65.

Iwasaki, S., and T. Takahashi. 1998. Developmental changes in calcium channel types mediating synaptic transmission in rat auditory brainstem. *J Physiol.* 509:419-23.

Johnson, M.I., D. Higgins, and M.D. Ard. 1989. Astrocytes induce dendritic development in cultured sympathetic neurons. *Brain Res Dev Brain Res.* 47:289-92.

Kang, J., L. Jiang, S.A. Goldman, and M. Nedergaard. 1998. Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat Neurosci.* 1:683-92.

Katz, L.C., and M. Constantine-Paton. 1988. Relationships between segregated afferents and postsynaptic neurones in the optic tectum of three-eyed frogs. *J Neurosci.* 8:3160-80.

Kimelberg, H.K., S.K. Goderie, S. Higman, S. Pang, and R.A. Waniewski. 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J Neurosci.* 10:1583-91.

Kossel, A., S. Lowel, and J. Bolz. 1995. Relationships between dendritic fields and functional architecture in striate cortex of normal and visually deprived cats. *J Neurosci.* 15:3913-26.

Kuwada, J.Y. 1986. Cell recognition by neuronal growth cones in a simple vertebrate embryo. *Science.* 233:740-6.

Largo, C., P. Cuevas, and O. Herreras. 1996a. Is glia disfunction the initial cause of neuronal death in ischemic penumbra? *Neurol Res.* 18:445-8.

Largo, C., P. Cuevas, G.G. Somjen, R. Martin del Rio, and O. Herreras. 1996b. The effect of depressing glial function in rat brain in situ on ion homeostasis, synaptic transmission, and neuron survival. *J Neurosci.* 16:1219-29.

Le Roux, P.D., and T.A. Reh. 1994. Regional differences in glial-derived factors that promote dendritic outgrowth from mouse cortical neurons in vitro. *J Neurosci.* 14:4639-55.

Li, Y.X., A.E. Schaffner, and J.L. Barker. 1999. Astrocytes regulate the developmental appearance of GABAergic and glutamatergic postsynaptic currents in cultured embryonic rat spinal neurons. *Eur J Neurosci.* 11:2537-51.

Liu, Q.Y., A.E. Schaffner, Y.H. Chang, K. Vaszil, and J.L. Barker. 1997. Astrocytes regulate amino acid receptor current densities in embryonic rat hippocampal neurons. *J Neurobiol.* 33:848-64.

Liu, Q.Y., A.E. Schaffner, Y.X. Li, V. Dunlap, and J.L. Barker. 1996. Upregulation of GABAA current by astrocytes in cultured embryonic rat hippocampal neurons. *J Neurosci.* 16:2912-23.

Matteoli, M., K. Takei, R. Cameron, P. Hurlbut, P.A. Johnston, T.C. Sudhof, R. Jahn, and P. De Camilli. 1991. Association of Rab3A with synaptic vesicles at late stages of the secretory pathway. *J Cell Biol.* 115:625-33.

Mennerick, S., and C.F. Zorumski. 1994. Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature.* 368:59-62.

Muller, C.M., and J. Best. 1989. Ocular dominance plasticity in adult cat visual cortex after transplantation of cultured astrocytes. *Nature.* 342:427-30.

Nakanishi, K., Y. Okouchi, T. Ueki, K. Asai, I. Isobe, Y.Z. Eksioglu, T. Kato, Y. Hasegawa, and Y. Kuroda. 1994. Astrocytic contribution to functioning synapse formation estimated by spontaneous neuronal intracellular Ca2+ oscillations. *Brain Res.* 659:169-78.

Noble, M., J. Fok-Seang, and J. Cohen. 1984. Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J Neurosci.* 4:1892-903.

O'Malley, E.K., B.A. Sieber, R.S. Morrison, I.B. Black, and C.F. Dreyfus. 1994. Nigral type I astrocytes release a soluble factor that increases dopaminergic neuron survival through mechanisms distinct from basic fibroblast growth factor. *Brain Res.* 647:83-90.

Orkand, R.K., J.G. Nicholls, and S.W. Kuffler. 1966. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J Neurophysiol.* 29:788-806.

Parpura, V., T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija, and P.G. Haydon. 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature*. 369:744-7.

Pasti, L., A. Volterra, T. Pozzan, and G. Carmignoto. 1997. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J Neurosci*. 17:7817-30.

Pfrieger, F.W., and B.B. A. 1996. Synapse formationin vitro requires a glia-derived signal. Abstract, Society for Neuroscience. Vol. 1996. 1949.

Pfrieger, F.W., and B.A. Barres. 1995. What the fly's glia tell the fly's brain. *Cell*. 83:671-4.

Pfrieger, F.W., and B.A. Barres. 1997. Synaptic efficacy enhanced by glial cells in vitro. *Science*. 277:1684-7.

Porter, J.T., and K.D. McCarthy. 1996. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. *J Neurosci*. 16:5073-81.

Raff, M.C., B.A. Barres, J.F. Burne, H.S. Coles, Y. Ishizaki, and M.D. Jacobson. 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science*. 262:695-700.

Rakic, P. 1990. Principles of neural cell migration. *Experientia*. 46:882-91.

Ramon y Cajal, S. 1911. Histologie du sisteme nerveux de l'homme et des vertebres. Maloine, Paris.

Rene, F., P. Poisbeau, C. Egles, R. Schlichter, and J.M. Felix. 1997. Co-culture of hypothalamic neurons and melanotrope cells: a model to study synaptogenesis between central neurons and endocrine cells. *Neuroscience*. 76:203-14.

Robitaille, R. 1998. Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. *Neuron*. 21:847-55.

Rosato Siri, M.D., and O.D. Uchitel. 1999. Calcium channels coupled to neurotransmitter release at neonatal rat neuromuscular junctions. *J Physiol.* 514:533-40.

Rouach, N., J. Glowinski, and C. Giaume. 2000. Activity-dependent neuronal control of gap-junctional communication in astrocytes. *J Cell Biol.* 149:1513-26.

Rouget, M., D. Araud, R. Seite, A. Prochiantz, and A. Autillo-Touati. 1993. Astrocyte-regulated synaptogenesis: an in vitro ultrastructural study. *Neurosci Lett.* 150:85-8.

Rousselet, A., L. Fetler, B. Chamak, and A. Prochiantz. 1988. Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev Biol.* 129:495-504.

Scholz, K.P., and R.J. Miller. 1995. Developmental changes in presynaptic calcium channels coupled to glutamate release in cultured rat hippocampal neurons. *J Neurosci.* 15:4612-7.

Scholz, K.P., and R.J. Miller. 1996. Presynaptic inhibition at excitatory hippocampal synapses: development and role of presynaptic Ca2+ channels. *J Neurophysiol.* 76:39-46.

Shatz, C.J. 1990. Impulse activity and the patterning of connections during CNS development. *Neuron.* 5:745-56.

Smith, G.M., U. Rutishauser, J. Silver, and R.H. Miller. 1990. Maturation of astrocytes in vitro alters the extent and molecular basis of neurite outgrowth. *Dev Biol.* 138:377-90.

Szatkowski, M., B. Barbour, and D. Attwell. 1990. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature.* 348:443-6.

Takeshima, T., K. Shimoda, Y. Sauve, and J.W. Commissiong. 1994. Astrocyte-dependent and -independent phases of the development and survival of rat embryonic day 14 mesencephalic, dopaminergic neurons in culture. *Neuroscience*. 60:809-23.

Tsacopoulos, M., and P.J. Magistretti. 1996. Metabolic coupling between glia and neurons. *J Neurosci*. 16:877-85.

Ullian, E., S.K. Sapperstein, K.S. Christopherson, and B.A. Barres. 2001. Control of Synapse Number by Glia. *Science*. 291:657-660.

van den Pol, A.N., and D.D. Spencer. 2000. Differential neurite growth on astrocyte substrates: interspecies facilitation in green fluorescent protein-transfected rat and human neurons. *Neuroscience*. 95:603-16.

Verderio, C., S. Coco, G. Fumagalli, and M. Matteoli. 1995. Calcium-dependent glutamate release during neuronal development and synaptogenesis: different involvement of omega-agatoxin IVA- and omega- conotoxin GVIA-sensitive channels. *Proc Natl Acad Sci U S A*. 92:6449-53.

Wang, L.C., D.H. Baird, M.E. Hatten, and C.A. Mason. 1994. Astroglial differentiation is required for support of neurite outgrowth. *J Neurosci*. 14:3195-207.

Wang, X.F., and M.S. Cynader. 1999. Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res Brain Res Protoc*. 4:209-16.

Wong, R.O., K. Herrmann, and C.J. Shatz. 1991. Remodeling of retinal ganglion cell dendrites in the absence of action potential activity. *J Neurobiol*. 22:685-97.

Wu, R.L., and M.E. Barish. 1994. Astroglial modulation of transient potassium current development in cultured mouse hippocampal neurons. *J Neurosci*. 14:1677-87.

Yuzaki, M., K. Mikoshiba, and Y. Kagawa. 1993. Cerebellar astrocytes specifically support the survival of Purkinje cells in culture. *Biochem Biophys Res Commun*. 197:123-9.

CHAPTER 3. EXTRACTION OF NEAR-FIELD FLUORESCENCE FROM COMPOSITE SIGNALS TO PROVIDE HIGH RESOLUTION IMAGES OF GLIAL CELLS

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Abstract

The sub-diffraction optical resolution that can be achieved using near-field optical microscopy has the potential to permit new approaches and insights into sub-cellular function and molecular dynamics. Despite the potential of this technology, it has been difficult to apply to cellular samples. One significant problem is that sample thickness causes the optical information to be comprised of a composite signal containing both near- and far-field fluorescence. To overcome this issue we have developed an approach in which a near-field optical fiber is translated toward the cell surface. The increase in fluorescence intensity during z-translation contains two components; a far-field fluorescence signal when the tip of the fiber is distant from the labeled cell, and combined near- and far-field fluorescence when the tip interacts with the cell surface. By fitting a regression curve to the far-field fluorescence intensity as the illumination aperture approaches the cell, it is possible to isolate near-field from far-field fluorescent signals. We demonstrate the ability to resolve actin filaments in chemically fixed, hydrated glial cells. A comparison of composite fluorescence signals with extracted near-field fluorescence demonstrates that this approach significantly increases the ability to detect sub-cellular structures at sub-diffraction resolution.

Introduction

The principle of near-field microscopy was first described in 1928 by Synge who suggested the possibility of extending the resolution of the light microscope by illuminating samples through a minute aperture that was significantly smaller than the wavelength of illuminating light. Ash & Nicholls (1972) put this method into practice using 3 cm radiation and a restricted aperture. Resolution equivalent to $\lambda/60$ was achieved. The use of a tapered optical fiber to provide restricted aperture illumination of samples has opened a new area of sub-diffraction resolution imaging (Betzig and Chichester, 1992). Using optical fibers with apertures of the order of 50 nm, it has been demonstrated that single molecules could be interrogated to determine their physico-chemical properties (Sanchez et al.1997; Xie and Dunn, 1994; Trautman et al.1997; Betzig and Chichester, 1992; Betzig and Chichester, 1993). Despite the potential for high resolution imaging that Near-Field Scanning Optical Microscopy (NSOM) provides, there have been few examples where this technique has been effectively utilized with biological specimens (Hwang et al.1998; Subramaniam et al.1998; Lewis et al.1999; Nagy et al.1999; Bui et al.1999; Marchese-Ragona and Haydon, 1997; Valaskovic et al.1997; Haydon et al.1996; Moers et al.1995; Hollars and Dunn, 1998; Sanchez et al.1997; Hwang et al.1995). Although those that have been successful demonstrate that NSOM has the potential to revolutionize the biosciences (Hwang et al.1998; Hollars and Dunn, 1998; Vickery and Dunn, 1999; Hwang et al.1995). The paucity of successful biological applications for NSOM probably arises because the original development of the technique was performed in the physical sciences where the samples have very different characteristics from those in the life sciences.

Instead of being a planar sample on a coverslip, as utilized in the physical sciences, cells have significant thickness, they must be imaged in solution, and finally, they are highly compliant samples. Consequently, despite repeated attempts to image living or even chemically fixed and hydrated cells, little success has been achieved.

Because of the potential breakthroughs in understanding cell biology that might be achieved if we could image with 50 nm resolution, we have taken a different approach to performing NSOM. In this report we describe the development of a Biological Near-Field Microscope (BNFM) which achieves sub-diffraction resolution on cells in solution.

Materials and Methods

Cell culture

Enriched astrocyte cultures were produced from 1-3 day old Sprague-Dawley rat cortices as previously described (Parpura et al.1994). Briefly, cortices were dissected and the tissue was enzymatically (papain 20 IU/ml; Sigma; 1 hr at 37°C) and mechanically dissociated. The cells were plated into culture flasks and maintained at 37 °C in a humidified 5 % CO_2 / 95 % room air. After 7-13 days in vitro (DIV) the cells were shaken twice, first for 1.5 h and following a change of media (cold) for 18 h on an orbital shaker at 260 RPM at 37 °C. The enriched astrocytes were detached from the culture flask by incubation with trypsin (0.01 %). The detached cells were diluted with MMEM, collected by centrifugation (10 minutes at 750 rpm at room temperature), re-suspended and plated onto poly-L-lysine coated 22 X 22 mm coverslips and maintained at 37 °C in a humidified 5 % CO_2 / 95 % room air.

Labeling of actin filaments

Cultured astrocytes were fixed with 4% formaldehyde in PBS for 30 minutes at room temperature. The cells were then rinsed with PBS, permeabilized with 0.25% triton X-100 PBS for 10 minutes and then blocked for 20 minutes with a PBS solution containing 5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3. Actin filaments were labels with two methods: Cells were exposed to Oregon Green 488 Phalloidin (Molecular Probes, 5U/ml in PBS with 0.5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3) for 20 minutes at room temperature. The staining solution was removed and the cells rinsed three times with PBS and were ready for imaging. Alternatively an anti-actin monoclonal antibody (Chemicon) was applied at a dilution of 1:100 in 0.5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3, overnight at 4°C. The cells were then rinsed three time (5 minutes) with 0.5% BSA, 5% normal goat serum, 0.25% Triton X-100 and 0.02% NaN_3, before application of a goat anti-mouse secondary antibody conjugated to Alexa fluo 488 (Molecular Probes) for one hour at room temperature. The preparations were then rinsed with PBS before imaging.

Confocal microscopy was performed using a Prairie Technologies (Middleton, Wisconsin) confocal microscope. Post-image processing was performed either with Adobe Photoshop v4.0, or using Metamorph software v4.1 (Universal Imaging Corp.)

Loading cells with calcein

To load cells with the indicator, calcein, astrocytes were incubated for 5 minutes in saline solution containing 5 μg/ml of calcein AM (Molecular Probes). This indicator is insensitive to ion concentrations, and is freely mobile in the cytosol.

Instrumentation

To perform our near-field studies we developed an integrated near-field illumination positioning source with a confocal detection pathway (NeD_{NF} Prairie Technologies, LLC, Middleton, WI) that is attached to a Nikon Diaphot 300 inverted microscope. Near-field optical fibers (50 nm reported aperture diameter, Topometrix, CA or Nanonics, Israel) were mounted on a triple axis motorized manipulator (25mm movement in each axis) which also contained three axes of piezo-controlled movement (15 μm in each axis). Optical fibers were positioned above cells by visualizing the relative position of cells and the optical fiber with an ORCA digital camera (Hamammatsu, Japan) that was connected to one arm of our optical detection pathway. Using this system we were able to control the position of the near-field fiber with respect to the sample using calibrated software in which we could define the x and y image coordinates, and then command the motors to drive the optical fiber to the appropriate location. An aperture was then positioned at the secondary image plane, around the image of the tip of the near-field fiber. Positioning was performed by 2 motors that are calibrated such that aperture positioning is controlled from the camera image. After confirming the appropriate positioning of the aperture, a mirror was moved out of the optical pathway so that all photons were directed to a photomultiplier tube. The PMT output was fed through a preamplifer to an integrator. To commence near-field studies we provided voltage ramps to the Z piezo to repeatedly extend the near-field fiber toward the sample.

Results

Previously we have demonstrated that the optical signal from a mobile fluorochrome within a cell can be used to monitor the relative positioning of a near-field optical fiber on a cell surface (Haydon et al.1996). In our previous studies we performed measurements at single points of the cell. We have now extended this approach to permit linescan and imaging modalities. As an initial test of our system, we loaded astrocytes with the freely mobile dye, calcein, and asked whether we could reliably detect a fluorescence signal as the optical fiber was translated in the z-axis towards the cell. Figure 1 shows a wide-field image of cultured astrocytes loaded with calcein. We then switched to illumination through the near-field optical fiber, and extended the fiber towards the cell. Figure 1b shows a typical fluorescence approach curve obtained during translation of the near-field fiber towards the cell. Note, that this fluorescence signal is a composite signal of both far-field and near-field fluorescence. Initially the fluorescence signal is due to far-field illumination of the sample.

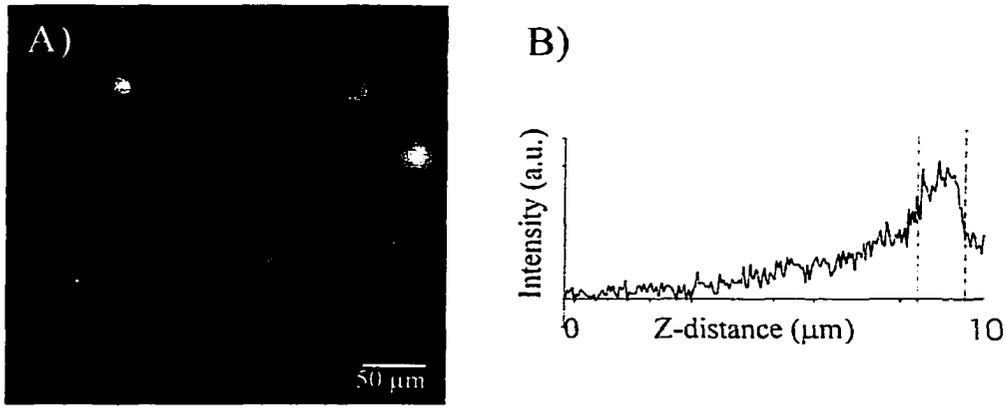

Figure 1. Translation of a near-field optical fiber to a calcein-loaded living glial cell demonstrates multiple fluorescent components. A) shows a photomicrograph of a field of glial cells that are loaded with the indicator calcein. B) depicts the relation between the fluorescent intensity of calcein and the z-position of a near-field optical fiber that provides the excitation illumination. As the fiber approaches the cell from a distance, the intensity of fluorescence increases due to far-field illumination. However, as the tip of the optical fiber locally interacts with the cell surface, there is a non-linear 'step' in fluorescent intensity (vertical dashed line) due to local excitation of the fluorophore by the evanescent wave of the optical fiber. Continued translation of the fiber causes little change in intensity until an anomalous reduction in intensity as the cell is compressed.

However, as the fiber made contact with the cell surface, there was a non-linear increase in fluorescence intensity, due to local excitation of the sample by the evanescent wave at the tip of the near-field optical fiber. Continued extension of the piezo caused no further increase in fluorescence since the tip of the fiber is in contact with the cell surface. However, as reported previously (Haydon et al.1996), when the piezo was extended for more than about one micrometer after contact we observe an anomalous reduction in intensity during excessive translation of the fiber. The cause of this reduction in fluorescence intensity is not clear, but may result, in part, from a compression of the cell as well as from a disturbance of the evanescent wave at the tip of the optical fiber. For the purposes of this study, however, we have ignored this anomalous reduction in fluorescence, although we frequently extended the piezo to cause this reduction in intensity to serve as a reference point.

For biological studies, where samples have significant thickness, composite far-and near-field fluorescence signals will be common place, and will hinder the potential resolution that can be achieved even with the smallest near-field aperture. To further test the utility of this approach we turned to chemically fixed astrocytes in which the actin cytoskeletal network was labeled with Oregon green 488 phalloidin. In contrast to calcein-loaded cells, phalloidin-labeled actin filaments provide a non-uniform fluorescent sample (Figure 2a). Z translation of a near-field optical probe to the surface of phalloidin-labeled astrocyte generates two types of fluorescence intensity curves. In the first, there is a relatively linear increase in fluorescence intensity as the tip approaches the cell surface (Figure 2b). In the second, there is this linear increase in far-field fluorescence until the fiber tip locally excites a phalloidin-labeled actin filament. Local excitation of the phalloidin-labeled actin filament by the evanescent wave causes a non-linear increase in fluorescent intensity (Figure 2c). Since the increase in far-field fluorescence, during z translation of the optical fiber, is approximately linear, we reasoned that we could extract the far-field from the near-field fluorescence by using a linear regression curve. Consequently, for each z translation we fit a linear regression curve to the data based on the slope calculated during the initial far-field fluorescence component. Figure 2c demonstrates that this approach does allow the selection of the non-linear fluorescence component from the fluorescence curve that is acquired during translation of the optical fiber to the cell surface. Since this non-linear component is not

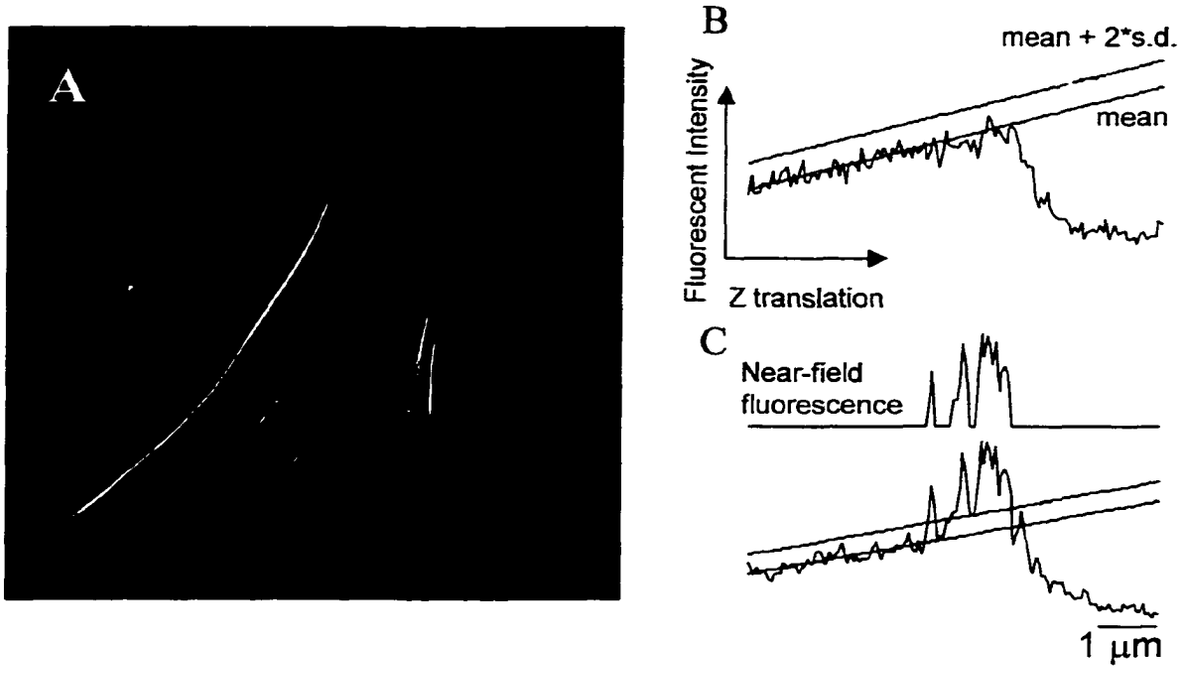

Figure 2. Oregon Green 488 phalloidin labeled actin filaments can be detected with near-field optical fibers. A), shows a top view reconstructed confocal image of a glial cell that is labeled with Oregon green 488 phalloidin. B) and C) show the relation between the fluorescent intensity of Oregon Green 488 phalloidin and the z-position of a near-field optical fiber. In B) the fluorescence signal is shown when a near-field fiber approaches a region of an astrocyte that does not contain a labeled actin filament beneath the tip of the near-field optical fiber. Only a linear increase in fluorescence intensity due to far-field excitation is detected. By contrast, (C), a step increase is fluorescence is detected when the evanescent wave of the near-field optical fiber illuminates a labeled actin filament. Superimposed on the data are two regressions corresponding to the linear regression of a baseline fit, as well as a linear regression that is offset by 2x standard deviations from the baseline. Note that as the tip interacts with the sample the non-linear increase in fluorescence exceeds these regressions. The upper trace represents the near-field fluorescence that was extracted from the composite signal (lower trace). Points that exceed the linear regression that corresponds to the mean plus 2 standard deviations for three consecutive points, were identified as due to evanescent excitation of the sample. While one might normally use 3 standard deviations as a criterion, we found that data which exceeded the mean plus 2 standard deviations for three consecutive points was a more stringent way of isolating near-field data since it prevented large transient 'spikes' being accepted as near-field data.

detected in unlabelled cells, is only obtained when the fiber approaches a labeled filament (as opposed to unlabelled regions of a cell) and since near-field fibers with a small aperture are required to detect this fluorescence, we suggest that it results from the local interaction of the evanescent component of the energy present at the tip of near-field optical fibers with the phalloidin-labeled actin filament

To further study the utility of the extraction of near-field from far-field fluorescence data, we performed linescans on phalloidin-labeled astrocytes. Figure 3 demonstrates the results of one such experiment. In this experiment, the z piezo was translated towards the cell and the peak fluorescence intensity (composite near- and far-field fluorescence signal), as well as the extracted near-field fluorescence intensities, were determined. Then the x piezo was used to move the fiber to an adjacent region before a second z translation was performed. By repeatedly performing z tranlations of the optical fiber that were followed by moving an x increment (Figure 3a), we were able to generate intensities of fluorescence at each point on a linescan (Figure 3b). In the composite fluorescence signal (Figure 3c) little structural definition can be resolved due to the overwhelming contribution of far-field fluorescence to the composite

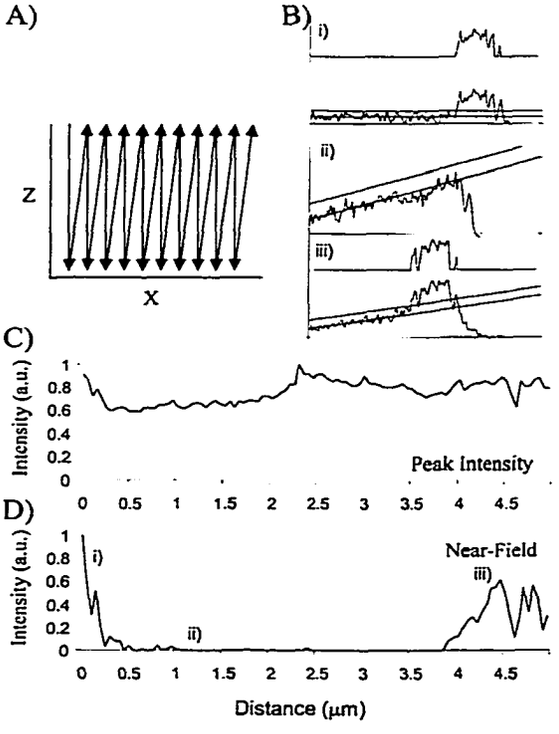

Figure 3. Linescan over bundles of actin filaments. A) To perform a linescan over the surface of an Oregon green 488 phalloidin labeled glial cell, the near-field fiber was translated in the z axis at each point along the line. In B) three z-translation fluorescence curves are shown at three points along the line (labeled i, ii, and iii). In C) the peak fluorescence at each point is shown, while in D) the intensity of the extracted near-field signal is shown. Note that the composite fluorescence signal in C) shows little resolution of actin filaments, whereas after extraction of the near-field signal, distinct actin bundles can be resolved (D).

signal. However when the near-field fluorescence component is extracted (Figure 3b) during each z-translation, and plotted on a normalized near-field linescan plot (Figure 3d), cross sections of bundles of actin filament are now resolved. Using this method it has been possible to resolve actin filaments with a resolution of about 50 nm (Figure 4). Further validation of the utility of this approach is provided by a rare experiment in which the size of the aperture increased while performing a linescan. When the size of the aperture increased it was difficult to detect the non-linear fluorescence component during z translations of the optical fiber and we were unable to detect structures with sub-diffraction resolution.

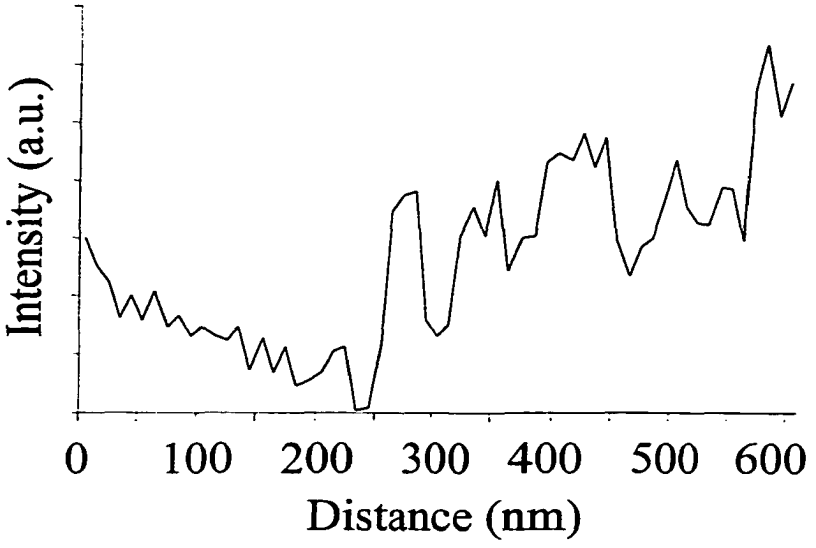

Figure 4. Linescan showing extracted near-field fluorescence intensity obtained from phalloidin-labeled astrocyte. A linescan was performed on a phalloidin-labeled astrocyte by performing sequential z-translations of the near-field optical fiber at 10 nm increments along the cell surface. Near-field fluorescence was extracted from total fluorescence using the linear regression method and is plotted at each x position. Intensities of fluorescence are shown on a linear scale with arbitrary units.

As a final test of this approach we have adopted an imaging mode in which each pixel in the x and y coordinates is generated by z translations of the optical fiber. Figure 5 shows an image of the peak fluorescence intensity (composite signal, Figure 5a) at each x, and y pixel during a near-field imaging experiment, and an image of the extracted near-field

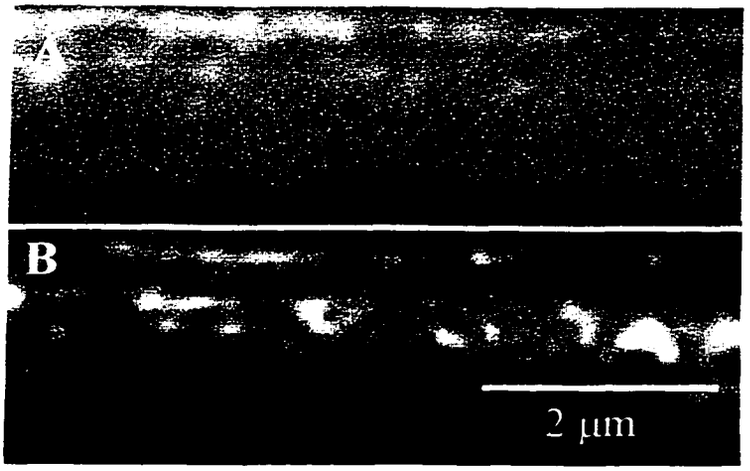

Figure 5. Composite fluorescence and near-field imaging of actin bundles. A and B) show 2x5 μm images that were generated over one actin bundle using a near-field optical fiber as the excitation source. In A) the peak intensity of fluorescence is shown (composite of near and far-field fluorescence) while in B) the extracted near-field fluorescence image is presented. In these images a 3x3 gaussian filter was used for display purposes

component of each pixel (Figure 5b). Clearly the utilization of a near-field extraction algorithm shows promise for permitting high resolution imaging of biological samples.

Discussion

In this study we have taken a novel approach to obtaining near-field optical data from cells. Instead of using shear-force feedback to maintain the near-field tip at a constant position with respect to the sample, we performed open loop experiments in which feedback *per se*, is not employed. Rather we use the fluorescent signal of the sample to provide the necessary information to obtain sub-diffraction resolution structural data. Using both living and chemically fixed (hydrated) astrocytes, our studies show that the fluorescent signal generated by a near-field optical fiber is a composite of both far-field and near-field fluorescence. While studies performed with single molecules or monolayers will not detect such a problem, this is a serious issue for biological imaging. Since cells have significant thickness, excitation energy that propagates to the far-field will illuminate distant fluorophore causing a high background fluorescence signal upon which the near-field signal is superimposed. If this signal were constant one could simply subtract a background level from a composite image to leave an uncontaminated near-field image. However, since this far-field component changes unpredictably across a cell, this approach is not feasible.

Instead we demonstrate an alternative approach in which the relative contribution from far- and near-field fluorescence signals are identified at each pixel.

To identify the near-field component of a cellular fluorescence signal we construct approach curves in which the slope of the far-field fluorescence, during the z-translation of the near-field field optical fiber, is used to identify the relative near and far-field signals (Figures 1-4). The validity of this method is demonstrated in several manners. We were unable to extract high-resolution near-field fluorescence when the aperture spontaneously enlarged during an experiment. When actin filaments were fluorescently labeled, z-translation of the optical fiber onto cells generated fluorescence curves consisting of either the far-field fluorescence (e.g. Figure 2b), or the composite near- and far-field fluorescence (e.g. Figure 2c). Finally, when a relatively homogeneously distributed fluorescent indicator, calcein, was loaded into astrocytes, we reliably detected both the near-field and far-field signals during each z-translation of the optical fiber, irrespective of its position over the cell.

One limitation of translating the near-field optical probe repetitively to the cell surface, in its current mode of operation, is that it is a relatively slow process that can take about 300 ms for each extension/retraction cycle. It should, however, be possible to increase the imaging speed by using higher vibration speeds and a lock-in approach in which the fluorescent intensity at two phases of the oscillation are taken as indications of the near-field and far-field fluorescence. With such an approach we anticipate that it should be possible to generate 100x100 pixel images in about 10 seconds.

Much care and attention has been taken in developing high speed, reliable feedback methods to control the positioning of near-field optical fibers over samples. While these feedback methods are critical for studies in the physical sciences that employ hard samples, we now demonstrate that they are not necessary for studying living cells. One major difference between these two classes of study is the compliance of the sample under study. In the original studies in the physical sciences, contact between the tip and substrate instantly damages the aperture of the near-field optical fiber, immediately compromising optical resolution. However, since cells are soft, contact between the cell surface and the optical fiber rarely leads to aperture damage.

Our study of the use of near-field optical fibers to image living cells should now pave the way for a series of novel high-resolution investigations in cell and molecular biology. Since we can image at sub-diffraction resolution, one can potentially investigate the distribution of membrane proteins and receptors, as well as other macromolecular entities. Perhaps even more exciting, however, is the opportunity to examine dynamic events in the cell cortex by illumination through the plasma membrane. This method will not, however, be able to probe at distances into the cell in a manner similar to confocal microscopy since the nature of the evanescent wave restricts biological near-field microscopy to surface imaging. Nonetheless for these surface measurements, near-field microscopy clearly surpasses confocal microscopy in x, y and z resolution. We have now demonstrated that we can reliably position near-field optical fibers on living cells, and in parallel studies, have demonstrated that the fibers are non-invasive to the cells. Using the near-field extraction algorithm that has been developed in this study, it is anticipated that we will be able to record highly localized sub-membrane changes in ion concentration when using fluorescent ion indicators. While evanescent wave or total internal reflection microscopy also reveals surface events such as vesicle dynamics (Zenisek et al., 2000), illumination through the near-field fiber provides increased x, y resolution, albeit at the expense of temporal resolution in imaging applications.

References:

Ash, E.A. and G. Nicholls. 1972 Super-resolution aperture scanning microscope. *Nature* 237:510-512.

Betzig, E. and R.J. Chichester. 1992. Near-field optics: microscopy, spectroscopy, and surface modification beyond the diffraction limit. *Science* 257:189-195.

Betzig, E. and R.J. Chichester. 1993. Single molecules observed by near-field scanning optical microscopy. *Science* 262:1422-1425.

Bui, J.D., T. Zelles, H.J. Lou, V.L. Gallion, M.I. Phillips, and W. Tan. 1999. Probing intracellular dynamics in living cells with near-field optics. *J. Neurosci. Methods* 89:9-15.

Haydon, P.G., S. Marchese Ragona, T.A. Basarsky, M. Szulczewski, and M. McCloskey. 1996. Near-field confocal optical spectroscopy (NCOS): subdiffraction optical resolution for biological systems. *J. Microsc.* 182:208-216.

Hollars, C.W. and R.C. Dunn. 1998. Submicron structure in L-alpha-dipalmitoylphosphatidylcholine monolayers and bilayers probed with confocal, atomic force, and near-field microscopy. *Biophys. J.* 75:342-353.

Hwang, J., L.K. Tamm, Bohm, T.S. Ramalingam, E. Betzig, and M. Edidin. 1995. Nanoscale complexity of phospholipid monolayers investigated by near-field scanning optical microscopy. *Science* 270:610-614.

Hwang, J., L.A. Gheber, L. Margolis, and M. Edidin. 1998. Domains in cell plasma membranes investigated by near-field scanning optical microscopy. *Biophys. J.* 74:2184-2190.

Lewis, A., A. Radko, N. Ben Ami, D. Palanker, and K. Lieberman. 1999. Near-field scanning optical microscopy in cell biology. *Trends. Cell Biol.* 9:70-73.

Marchese-Ragona, S.P. and P.G. Haydon. 1997. Near-field scanning optical microscopy and near-field confocal optical spectroscopy: emerging techniques in biology. *Ann. N. Y. Acad. Sci.* 820:196-206; discussion 206-.

Moers, M.H.P., A.G.T. Ruiter, A. Jalocha, and N.F. Van Hulst. 1995. Detection of fluorescence in situ hybridization on human metaphase chromosomes by near-field scanning optical microscopy. *Ultramicroscopy* 61:279-283.

Nagy, P., A. Jenei, A.K. Kirsch, J. Szollosi, S. Damjanovich, and T.M. Jovin. 1999. Activation-dependent clustering of the erbB2 receptor tyrosine kinase detected by scanning near-field optical microscopy. *J. Cell Sci.* 112:1733-1741.

Parpura, V., T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija, and P.G. Haydon. 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747.

Sanchez, E.J., L. Novotny, G.R. Holtom, and X.S. Xie. 1997. Room-temperature fluorescence imaging and spectroscopy of single molecules by two-photon excitation. *J. Phys. Chem.* 101A:7019-7023.

Subramaniam, V., A.K. Kirsch, and T.M. Jovin. 1998. Cell biological applications of scanning near-field optical microscopy (SNOM). *Cell Mol. Biol. (Noisy-le-grand).* 44:689-700.

Synge, E.H. 1928 A suggested method for extending microscopic resolution into the ultramicroscopic region. *Phil.Mag.* 6:356-362

Trautman, J.K., J.J. Macklin, L.E. Brus, and E. Betzig. 1994. Near-field spectroscopy of single molecules at room temperature. *Nature* 369:40-42.

Valaskovic, G.A., M. Holton, and G.H. Morrison. 1997. Biological near-field scanning optical microscopy: instrumentation and sample issues for shear-force feedback. *Ultramicroscopy* 57:212-218.

Vickery, S.A. and R.C. Dunn. 1999. Scanning near-field fluorescence resonance energy transfer microscopy. *Biophys. J.* 76:1812-1818.

Xie, X.S. and R.C. Dunn. 1994. Probing single molecule dynamics. *Science* 265:361-364.

Zenisek, D., J.A., Steyer and W. Almers. 2000. Transport, capture and exocytosis of single synaptic vesicles at active zones. *Nature* 406: 849-854.

CHAPTER 4. GENERAL SUMMARY

This dissertation presents the results of an investigation into the effects of astrocytes on hippocampal synapse formation and calcium current function *in vitro* and the development of a new microscope for near-field imaging of biological samples.

Understanding the role glial cells play in central nervous system function has been expanding at an increasing pace. This research adds to that body of knowledge by showing that astrocytes accelerate the formation of synapses in cultured hippocampal neurons. Synapses formed in the presence of astrocytes were more numerous, had increased dendritic complexity, contained more ultrastructurally mature profiles, had more rapid delivery of synaptic vesicle protein, synaptotagmin I, to the processes and experienced a significant increase in the N-type calcium current at the time when synapses were forming.

Electrophysiologically, there was significant up-regulation of excitatory synapse formation in astrocyte-enriched cultures as assessed by both spontaneous and evoked synaptic responses. Also, a dramatic effect of astrocyte contact was documented on the behavior of N-type calcium currents, with an average 11-fold increase in the current when the neuronal soma was in contact with an astrocyte.

The second portion of this dissertation describes a near-field microscope specifically designed for biological samples. Imaging within the near-field of a point source of illumination allows resolution of the sample below the limits of normal optical microscopy. The major limitation to the application of near-field microscopy to biology to date is the physical presentation of the sample. Biological samples have extremes of topography and are soft as opposed to material science samples that tend to be more or less uniform.

The near-field microscope described delivers the excitation illumination through a fine-tipped fiber optic that is moved in relation to the sample. The excitation light is delivered from above as the fiber is moved toward and ultimately contacts the sample. Emission from the sample is collected from below with a high quality microscope objective and delivered to a photo-multiplier tube for detection. The signal is captured and analyzed by software that performs an extraction of the near-field data from the contaminating far-field illumination.

The microscope can be operated in three modalities. Single point, the fiber can monitor a specific point on the sample to record rapid fluorescence changes. Line scan mode allows investigation of a defined two-dimensional portion of the sample. Imaging the sample can be accomplished by x and y translation of the fiber to build up a pixel-by-pixel image of the region of interest.

APPENDIX A. TARGET-DEPENDENT INDUCTION OF SECRETORY CAPABILITIES IN AN IDENTIFIED MOTORNEURON DURING SYNAPTOGENESIS

Target- Dependent Induction of Secretory Capabilities in an Identified Motoneuron during Synaptogenesis

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Cholinergic neurons isolated from the buccal ganglia of *Helisoma* were plated into cell culture with a variety of defined target cells to study the specificity of synaptogenesis. Motoneuron B19 selectively formed chemical connections with single dissociated muscle fibers derived from its appropriate target, the supralateral radular tensor (SLT) muscle. B19 did not form such connections with novel neuronal targets. In contrast to neuron B19, cholinergic neuron B5 nonselectively formed chemical connections with novel muscle and neuronal targets. Target cells were micromanipulated into contact with presynaptic neurons to examine the latent period until the onset of functional synaptic transmission. Neuron B5 formed chemical connections within the first minutes of contact with ACh-sensitive neurons and muscle while B19 required sustained periods of muscle-specific contact to induce the acquisition of a functional excitation-secretion coupling mechanism. These different latent periods from the onset of target contact suggest that neuron B5 acquires presynaptic secretory function before target contact, while B19 must receive a specific signal(s) from its appropriate target to induce the transformation of its terminal into a secretory state.

INTRODUCTION

Formation of specific synaptic connections is a fundamental feature of neural development. Studies of neurons and targets cells in culture have contributed to our understanding of synapse formation in vertebrates (Frank and Fischbach, 1979; Kidokoro and Yeh, 1982; Hume et at, 1983; Xie and Poo, 1986; Chow and Poo, 1985; Role et at, 1987; Evers et at, 1989) and invertebrates (Ready and Nicholls, 1979; Fuchs et al., 1981; Hadley et at, 1983; Bodmer et at, 1984; Ambron et at, 1985; Schacher et at, 1985; Arechiga et at, 1986; Chiquet and Nicholls, 1987; Haydon and Kater, 1988; Haydon et al., 1990). During normal neural development, many factors govern the formation of specific synaptic connections such as temporal sequences of neurogenesis, neurite guidance and pathfinding, trophic effects,

cellcell interactions, synaptic competition between neurons, and synapse elimination. Using in vitro studies of synaptogenesis, it is possible to examine the importance of cell-cell recognition in the process of synaptogenesis (Lin and Levitan, 1987; Vyklicky and Nicholls, 1988; Haydon and Zoran, 1989; Evers et at, 1989; Buchanan et at, 1989).

When identified neurons have been plated into cell culture and allowed to contact, electrophysiological studies have shown that certain cells are selective in synaptogenesis, while others are unconstrained and form novel connections (Camardo et at, 1983; Schacher et at, 1985; Arechiga et at, 1986; Haydon and Kater, 1988; Haydon and Zoran, 1989). Although little is known about the molecular mechanisms underlying such differences in synaptic specificity, it is clear that early cellular interactions (e.g., cell adhesion and recognition) are important (Chow and Poo, 1985; Sun and Poo, 1987; Takahashi et at, 1987; Evers et at, 1989; Haydon and Zoran, 1989).

Cholinergic neuron B5, isolated from the buccal ganglion of Helisoma and plated into neuronal culture, reliably forms the presynaptic element of novel chemical connections (Haydon, 1988; Haydon and Kater, 1988; Haydon and Zoran, 1989; Haydon and Zoran, 1990). In contrast to neuron B5, cholinergic motoneuron B19 from Helisoma (Zoran et at, 1989) is restricted in synaptogenesis and never forms the presynaptic element of novel chemical connections in cell culture (Haydon and Kater, 1988; Haydon and Zoran, 1989; Haydon and Zoran, 1990). Since action potential-evoked ACh release is not reliably detected when neuron B19 contacts ACh-sensitive targets (Haydon and Zoran, 1989), we hypothesize that this neuron might require recognition of an appropriate target to acquire the ability to release ACh. In this study, we have tested this hypothesis using neurons B19 cultured in contact with supralateral radular tensor (SLT) muscle fibers, the appropriate postsynaptic target of B19 (i.e., its normal synaptic connection in the unperturbed adult nervous system) (Zoran et al., 1989). Neuron B19, following contact with appropriate muscle targets, gains the ability to release neurotransmitter in response to presynaptic action potentials.

MATERIALS AND METHODS

Laboratory reared albino (red) *Helisoma trivolvis* were used in all experiments. These adult animals were fed lettuce and trout chow daily and were maintained at room temperature.

Neuronal Cell Cultures

Single specific neurons, identified according to size, position, pigmentation, and axonal configuration, were isolated from buccal ganglia that had been pretreated with 0.2% trypsin (Sigma type III) for 30 min (Hadley *et al.,* 1985; Haydon *et al.,* 1985). Neurons were transfered to poly-L-lysine-coated, 35-mm culture dishes (Falcon 3001) that contained 2 ml of conditioned medium (CM) (Wong *et al.,* 1981). Neurons plated under these culture conditions adhered to the substrate, formed motile growth cones, and extended neurites. Conditioned medium was obtained by incubating 10 ml of defined medium (DM) with 20 sterile brains (i.e., central ganglia) for 3 days. Brain-derived neurite outgrowth -promoting factors are released into the medium during this time. DM used for these cultures contained 50% Lebowitz-15 (GIBCO; special order 82-5154EL, without inorganic salts) to which L-glutamine (0.15 mg/ml of DM) and inorganic salts were added to give a final concentration of (in mM) 40 NaCl, 1.7 KCI, 1.5 MgC12, 4.1 CaC12, 10 Hepes, pH 7.4,130 mOsm.

Pairs of isolated neurons were cultured for 3 to 4 days to allow contacts to be made between outgrowing neurites of the adjacent cells. At this time, 5% *Helisoma* hemolymph, collected from adult animals according to the methods of Hadley and Kater (1983), was added to the cultures. Hemolymph, which promotes chemical synapse formation between snail neurons in cell culture (Haydon and Zoran, 1990), was not added until after establishment of cell-cell contact since *Helisoma* serum can have pronounced effects on neuritic architecture including net retraction of neuronal processes (Grega and Kater, 1987).

Electrophysiological assessment of functional connectivity was conducted within 8-24 hr after the addition of hemolymph for these sustained cell cultures. In the description of experimental results, we classify potential postsynaptic neurons as "target" cells to distinguish them from potential presynaptic cells. By using the term "target" we do not mean

to imply that such cells are normal targets *in* vivo. We use the terms "appropriate" and "novel" synaptic partners to identify cells that are and are not synaptically connected in the unperturbed adult nervous system, respectively.

Spherical Assay Somata

ACh-sensitive neuronal somata of neuron B19 were used in some experiments as assay cells for the detection of acetylcholine release from cultured cholinergic neurons following methodologies previously reported (Haydon and Man-Son-Hing, 1988; Haydon and Zoran,1989). Assay neurons were cultured for 2 days in 1% *Helisoma* hemolymph in DM, thereby preventing adhesion and neurite extension. Spherical somata of neuron B19 cultured in this fashion are sensitive assays of ACh release (Haydon, 1988; Haydon and Man-Son-Hing, 1988; Haydon and Zoran, 1989) and by penetrating somata with microelectrodes can be manipulated into contact with neuronal processes while monitoring the formation of chemical connections. Early events in synapse formation, during the first 30 min of target cell contact, were assessed using this technique.

Muscle Cell Dissociation

The supralateral radular tensor (SLT) muscle, innervated by neuron B19 *in vivo* (Zoran *et al.,* 1989), was dissected whole from approximately 20 animals and transferred through three 30-min incubations in DM. The SLT muscle masses were then incubated for 4-8 hr in 2 ml of DM containing 1% hemolymph and 4 mg of collagenase (Worthington Biochemical Corp.; CLS IV). The dissociation flask was gently agitated in a 34'C water bath. Following collagenase incubation, the tissue was washed several times at room temperature with DM and shaken by hand to dissociate the SLT muscle into single fibers. The dissociated fibers were collected by centrifugation and transferred to nonadhesive culture dishes for storage at room temperature. Muscle dissociation procedures have been previously described (Zoran *et al.,* 1989; Haydon and Zoran, 1990) where the physiological integrity of these preparations was established.

Single SLT muscle cells (100-1000 um in length and 5-15,um in width) maintained normal electrophysiological and contractile properties in cell culture, including

responsiveness to application of ACh (Zoran *et al.*, 1989). SLT fibers used in synaptogenesis experiments were plated adjacent to neurons in culture dishes for 3 to 4 days prior to the addition of 5% hemolymph following procedures described for neuronal cell cultures. These nerve-muscle cocultures were then electrophysiologically assessed for neuromuscular connectivity or were used together with neuronal assay somata to assess the influence of muscle on the neuron's ability to release neurotransmitter onto other targets.

Electrophysiology

Cells were visualized with phase-contrast optics using an inverted Olympus CK2 microscope. Glass microelectrodes, filled with 1.5 M KCI, were used for neuronal and muscle cell penetrations and had resistances of 10-20 and 30-80 MΩ respectively. The electrodes were connected to the bridge-balance amplifiers of a Dagan 8800 Total Clamp. The presence of chemical and electrical connections between neuron and neuron or neuron and muscle (chronically contacting) cell pairs were assessed using the criteria described under Results. Permanent records of this electrical activity were saved on chart recorder paper (Gould 220 Recorder).

In some experiments, ACh-sensitive neuronal somata and single muscle fibers were micromanipulated into contact with presynaptic neurons to determine whether chemical connections form after brief periods of contact (<30 min). The assay cells were impaled with microelectrodes as described above or were voltage clamped in the whole-cell mode with patch pipets filled with an internal solution consisting of 50 mM KCI, 5 MM MgC12, 5 mM EGTA, and 5 mM Hepes (pH 7.3). Patch pipets used in brief contact experiments had direct current resistances of 1-2 MΩ Records were collected with a Dagan 8900 patch-clamp amplifier and the data were stored on VHS tape using a Vetter 420F videotape recorder for later analysis.

In all experiments, the release of neurotransmitter was classified as being either spontaneous or evoked. Events were classified according to Evers *et al.* (1989) as spontaneous synaptic potentials (SSPs) or spontaneous synaptic currents (SSCs) when they were not correlated with action potentials in the presynaptic cell and as evoked synaptic potentials (ESPs) or evoked synaptic currents (ESCs) when elicited by action potentials in the presynaptic cell. Events were considered as evoked only if they followed presynaptic

action potentials at a constant latency within a given preparation. The presence of ESPs or ESCs was required to fulfill criteria for a functional chemical connection. For analyses of rates and amplitudes of synaptic currents, all events detected during a recording period were used in the calculation of mean values.

Pharmacological agents, such as tubocurarine chloride (Sigma), were perfused through the recording chamber using a gravity inflow system. All pharmacologic and ionic solutions were made in *Helisoma* DM. The zero calcium DM used in these studies contained 0 mM Ca" and 10 mM Mg".

RESULTS

Motoneuron B19 Selectively Forms Appropriate Chemical Connections with SLT Muscle Fibers

A known postsynaptic target of motoneuron B19 *in situ,* the supralateral radular tensor (SLT) muscle (Zoran *et al.,* 1989), was dissociated into single fibers and plated into culture with this motoneuron (Fig. 1). Neuron B19 extended neurites and contacted SLT muscle fibers after approximately 2 to 3 days of culture, the latency to contact depending largely on the time of initiation of neurite extension. Nerve-muscle cocultures were left for a further 3 to 4 days for neurite extension and the establishment of additional cell-cell contact.

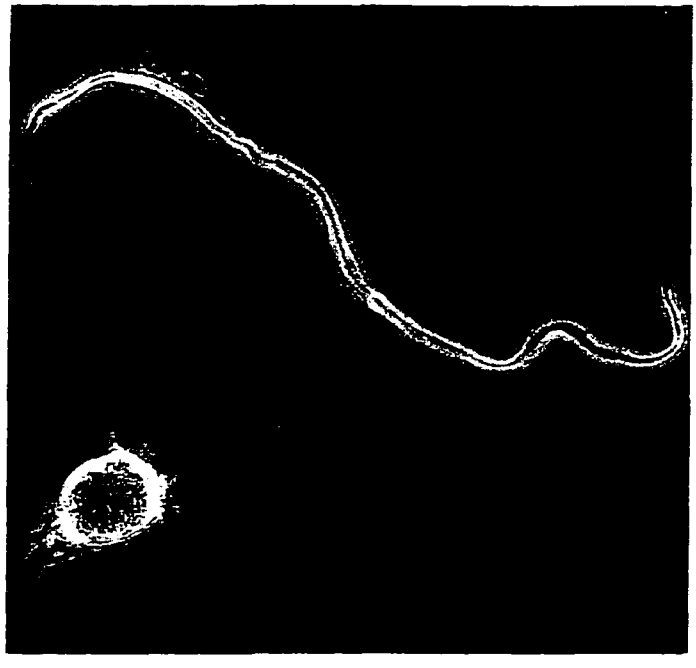

Fig. 1. Helisoma buccal nerve-muscle cocultures. A single neuron B19, isolated from the buccal ganglion (see Materials and Methods), extends neurites and contacts a dissociated supralateral radular tensor (SLT) muscle fiber in cell culture. Bar - 100,μM.

The cocultures were then incubated for 2-8 hr in 5% snail hemolymph (see Materials and Methods) prior to the electrophysiological assessment of synaptic connectivity. SLT muscle fibers cultured in contact with neuronal processes of motoneuron B19 formed the postsynaptic element of synaptic connections. The small diameter (5-15 μm) of the isolated SLT muscle fibers, together with their contractile nature, limited the duration of intracellular muscle recordings. Vigorous longitudinal contractions of neuron -contacted SLT fibers, but not control solitary fibers, resulted in muscle fibers tearing away from the polylysine-coated substrate during synaptogenesis *(n = 15* of 20 preparations). Such irreversible muscle fiber contractions prevented the electrophysiological assessment of synaptic connectivity at this time of culture. Nerve-muscle cocultures were tested for synaptic connectivity after 24-48 hr of contact to overcome this problem. At this stage of synapse formation, we would expect synapses to be less mature and the amplitudes of ESPs to be smaller.

Simultaneous nerve-muscle penetrations permitted assessment of synaptic connectivity in 35 B19-SLT preparations during early stages of synapse formation. Action potentials in B19 intermittently evoked depolarizing synaptic potentials in SLT muscle fibers during early times of cell contact (Fig. 2a). Such action potential-evoked synaptic potentials were detected in 17.1% of these B19-SLT neuromuscular cocultures examined *(n = 6* of 35). The amplitude of these ESPs was augmented by dc hyperpolarization of the muscle membrane potential *(n = 3* of 3). In addition, spontaneous synaptic potentials (SSPs) were detected in 34.3% of the B19-SLT preparations *(n = 35*; Fig. 2b). Injection of dc hyperpolarizing current into neuron B19 had no effect on the membrane potential of the postsynaptic muscle cells *(n = 34* of 35; Fig. 2a). Since muscle fiber recordings were short lived, it was not possible to apply pharmacological agents to the bathing medium to further test the chemical nature of these connections (although see below). Nonetheless, these results are consistent with the hypothesis that B19-evoked depolarizing synaptic potentials were chemical in nature (see also below).

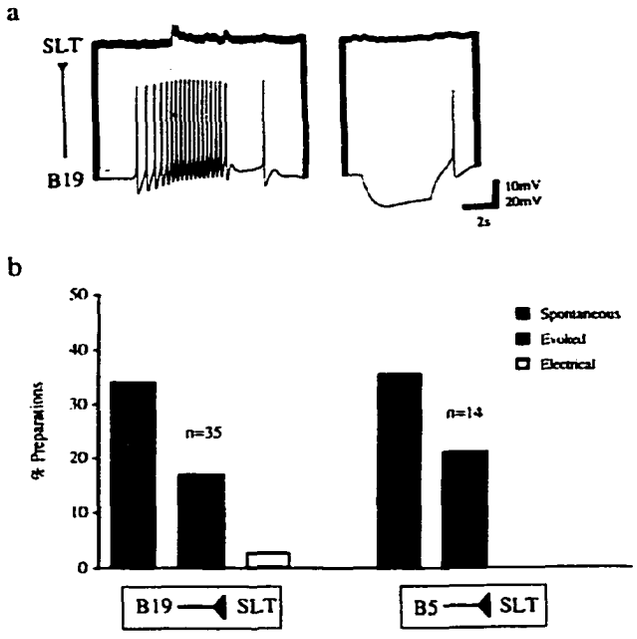

FIG. 2. Appropriate and novel neuromuscular synapse formation between buccal neurons and dissociated SLT muscle fibers. (a) Simultaneous intracellular recordings from a B19-SLT cell pair show that repetitive action potentials in presynaptic neuron B19 intermittently evoked synaptic potentials in its appropriate SLT muscle target (left traces). In the same preparation, injection of de hyperpolarizing current in the presynaptic B19 had no effect on the postsynaptic SLT muscle fiber (right traces). (b) Neuron B19 and neuron B5 form appropriate and novel chemical connections with SLT muscle fibers, respectively. Neither of the buccal neurons formed novel electrical connections with dissociated buccal muscle cells. Histograms represent the percentage of buccal neuron-SLT muscle fiber cocultures in which SSPs, ESPs, and electrical connections were detected.

Previous studies have failed to report the formation of chemical connections by B19 with novel targets (Haydon, 1988; Haydon and Kater, 1988; Haydon and Zoran, 1989). To test the extent to which neuron B19 is restricted in its ability to form chemical connections, novel neuronal targets were plated into culture togetherwith buccal neuron B19 and allowed to extend neuritis under identical conditions to those which permitted the formation of appropriate nerve-muscle connections. Intracellular recordings were made from pairs of neurons after 3 to 4 days in culture. Neuron B19 reliably formed electrical connections with novel and appropriate neuronal targets (Table 1; also Hadley and Kater,1983). Electrical

coupling, determined by injection of dc hyperpolarizing current into the presynaptic cell and

TABLE 1
PERCENTAGE OF CELL PAIR PREPARATIONS WITH FUNCTIONAL CHEMICAL TRANSMISSION AND ELECTRICAL COUPLING

Cell pair				
Pre	Post	% Chemical	% Electrical	Coupling coefficient
B19	B19	0 (n = 10)	100 (n = 10)†	0.31 ± 0.04
B19	B5	0 (n = 11)	100 (n = 11)*	0.34 ± 0.06
B19	SLT	17.1 (n = 35)†	2.9 (n = 35)*	0.09
B5	B5	87.5 (n = 8)*	100 (n = 8)*	0.25 ± 0.07
B5	B19	81.8 (n = 11)*	100 (n = 11)*	0.27 ± 0.05
B5	SLT	21.4 (n = 14)*	0 (n = 14)	0
SLT	SLT	—	46.2 (n = 13)	0.32 ± 0.03

† Denotes appropriate synaptic connection (see Materials and Methods).
* Denotes novel synaptic connection.

monitoring voltage responses in the postsynaptic cell, was detected in 100% of the B19-B19 (Fig. 3a; *n = 10;* coupling coefficient (Vpost,/Vpre) = 0.31 ± 0.04; mean ± sem) and B19-B5 *(n = 11;* coupling coefficient = 0.34 ± 0.06) cell pairs tested. B19 action potential-evoked synaptic potentials in nerve-nerve cocultures were single component, short duration electrical potentials which were unaffected by 10-5 M tubocurarine *(n = 3* of 3) or 0 Ca' medium *(n = 2* of 2). Evoked release of neurotransmitter was never detected from neuron B19 when paired in culture with cholinoceptive neurons B5 or B19 (Table 1). SSPs were occasionally recorded in novel target cells *(n = 2* of 21 preparations; both B5s). These results further indicated that presynaptic neuron B19, although competent to form chemical connections with appropriate SLT muscle targets, was unable to form chemical connections with novel neuronal targets.

Cholinergic Neuron B5 Indiscriminately Forms Chemical Connections with Novel Neuronal and Muscle Targets

Contrary to results obtained with neuron B19, buccal neuron B5 reliably forms chemical connections with novel ACh-sensitive targets (Haydon, 1988; Haydon and Kater, 1988; Haydon and Zoran, 1989). Neuron B5 reliably formed cholinergic connections with novel neuron targets B5 (Fig. 3b) and B19 (Fig. 3c). In these preparations, action potentials evoked two-component, electrical and chemical synaptic potentials as described previously by Haydon and Kater (1988). Typically, action potentials evoked both electrical and chemical synaptic potentials in a one-for-one manner. In some preparations, the chemical, but not the electrical, component of the ESP was evoked only intermittently (Fig. 3c).

Chemical ESPs were augmented by postsynaptic hyperpolarization *(n = 3* of 3), reduced in amplitude by 10,uM tubocurarine *(n = 6* of 6; Fig. 3c), and blocked by 0 Ca" DM *(n = 4* of 4). Chemical connections were detected in 87.5% of B5-B5 cell pairs *(n = 8)* and 81.8% of all B5-B19 cell pairs *(n = 11)* tested (see Table 1). All of these preparations were electrically coupled and had coupling coefficients of 0.25 ± 0.07 (mean ± sem) for B5-B5 pairs and 0.27 ± 0.05 for B5-B19 pairs.

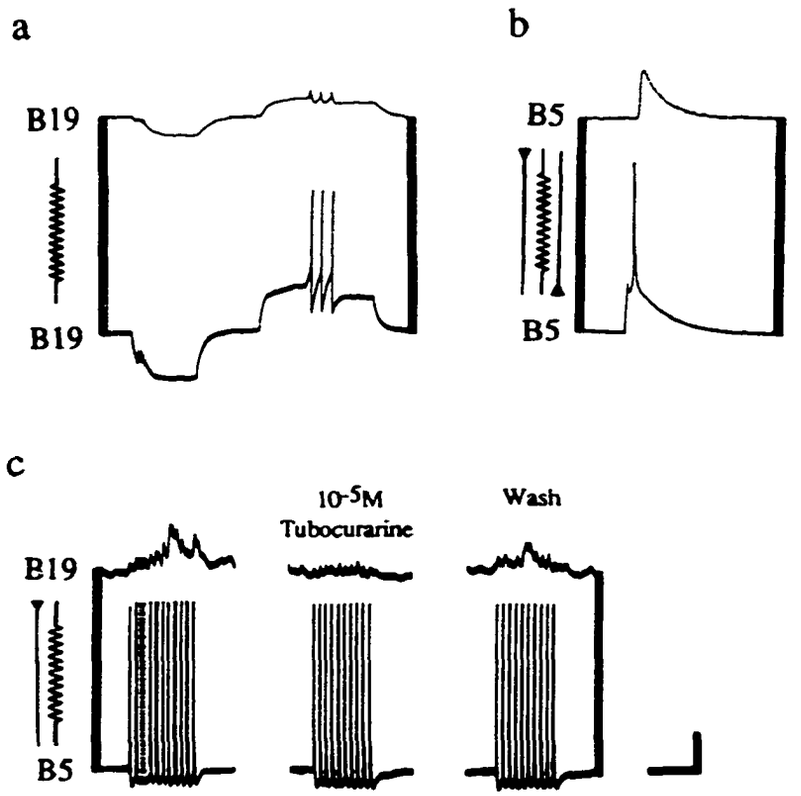

Fig. 3. Appropriate and novel synaptic connections formed by *Helisoma* buccal neurons in cell culture. (a) Neuron B19 forms appropriate electrical synaptic connections with a partner B19 recorded after 3 days in coculture. Injection of de hyperpolarizing current into the presynaptic neuron (bottom trace) causes a simultaneous hyperpolarization in the target B19 (top trace). Action potentials in B19 evoke short-latency electrical ESPs in postsynaptic neuron B19. No evidence of chemical connections was detected in 1319-1319 cell pairs. (b) Neuron B5 plated into coculture with another B5, forms novel chemical and electrical connections. Action potentials in presynaptic B5 of this cell pair produced one-for-one ESPs in the target B5 (c) Neuron B5 also formed novel chemical and electrical connections with target neuron B19 in 3-day cocultures. Action potentials in this preparation produced intermittent, two-component synaptic potentials. The cholinergic component of this ESPs was reversibly reduced by addition of 10-5 M tubocurarine to the bathing medium unmasking the electrical portion of the ESP. Vertical scale = 20 mV (a, top and bottom trace; b and c, bottom trace) and 10 mV (b and c, b top trace). Horizontal scale - 2 see (a and c) and 0.4 see (b).

Under the same culture conditions used for B19-SLT connectivity studies, neuron B5 was capable of forming novel chemical connections with dissociated SLT muscle fibers (Fig. 2b). SSPs and ESPs were detected in 35.7 and 21.4% of the B5-muscle preparations *(n = 14)*, respectively. Taken together, these results show that neuron B5 is capable of forming chemical connections with novel neuronal and muscle targets, while neuron B19 forms chemical connections with its appropriate muscle target only.

Electrical coupling was never detected between neuron B5 and SLT muscle fibers plated together into cell culture *(n = 14*; Table 1). The incompetence of buccal neurons B5 and B19 to reliably form electrical connections with SLT muscle fibers under these cell culture conditions was consistent with the lack of electrical connections detected between motoneuron B19 and its target SLT muscles *in situ* (Zoran *et al.*, 1989). The absence of electrical synapse formation between buccal neurons and SLT muscle fibers in cell culture was unexpected since both neurons B5 and B19 readily form electrical connections with other neuronal partners (Table 1; also Hadley and Kater, 1983; Hadley *et al.*, 1985; Haydon and Kater, 1988). This might indicate an incompatibility between nerve and muscle cells for electrical synapse formation. Alternatively, the lack of neuromuscular electrical connections could be explained by a general incompetence of muscle cells to form electrical connections. Experiments performed with SLT-SLT muscle cell pairs, however, reject this possibility (Haydon and Zoran, 1990). SLT muscle fibers, cultured in contact with each other for 2 days under identical conditions to those described for the previous nerve-nerve and nerve-muscle studies, were electrically coupled in 46.2% of the cell pairs tested *(n* = 13; Table 1). Coupling coefficients in these SLT-SLT muscle cell pairs were 0.32 ± 0.03 (mean ± sem).

Neuron B5, but Not B19, Forms Chemical Connections with SLT Muscle after Brief Periods of Contact

Previous studies (Haydon and Man-Son-Hing, 1988; Haydon and Zoran, 1989) have shown that neuron B5, but not neuron B19, can form chemical connections with novel neuronal targets after brief periods of contact. Action potentials in neuron B5 elicited ESPs in target

cells within the first minute of contact in 56% of all preparations that formed synaptic connections (Haydon and Zoran, 1989a). Since both B19 and B5 formed chemical connections when cocultured with SLT muscle fibers (Fig. 2b), we determined whether both of these connections would form rapidly. Single SLT muscle fibers were either penetrated with a microelectrode or voltage clamped in the whole-cell mode with a patch pipet and were then micromanipulated up to a neuronal process and growth cone (Fig. 4a). The timing of the onset of chemical synapse formation was then examined over the first 30 min of cell contact.

Figures 4b and 4d show that action potential-evoked release of neurotransmitter was never detected between briefly contacting neurons B19 and SLT muscle fibers (0 of 15 growth cones; $n = 15$ preparations). However, SSPs were detected from 33.3% of these neuron B19s ($n = 15$ preparations; Figs. 4b and 4d). In contrast to B19, both SSPs (28.6%) and ESPs (28.6%) were detected in SLT muscle fibers following contact with growth cones of neurite-bearing neuron B5 ($n = 7$; Figs. 4c and 4d). Electrical coupling was not detected in any of these B19 or B5-muscle micromanipulation studies (Figs. 4b and 4c). Thus, B5 but not B19, can form functional chemical connections with SLT muscle fibers after brief periods of contact.

Induction of Secretary Properties in Neuron B19 by Contact with SLT Muscle

A perplexing aspect of these studies with SLT muscle fibers is that chemical connections were detected in only a small percentage of the preparations examined (e.g., 21.4% of B5-SLT cocultures), while such connections were reliably detected between pairs of neurons (e.g., 87.5% of B5-B5 pairs; Table 1). Perhaps ACh sensitivity or adhesivity of the muscle cells may be decreased during the long enzymatic digestion during dissociation.

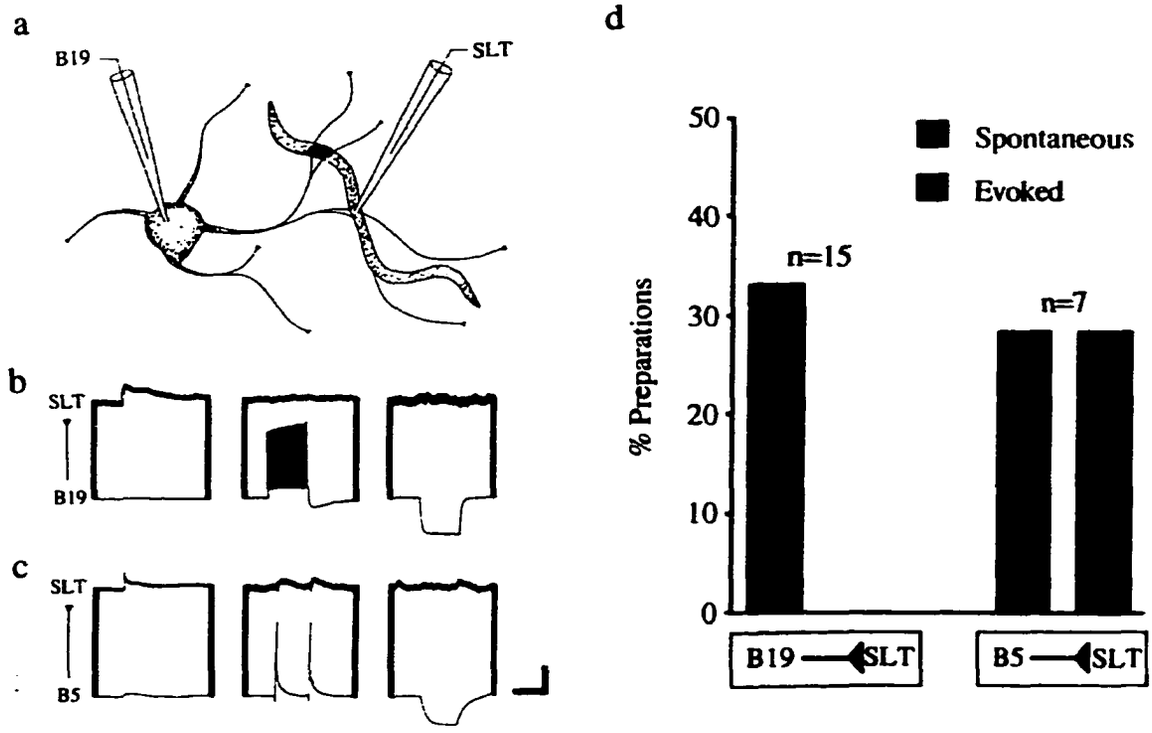

FIG. 4. Effect of brief periods of SLT muscle contact on synaptic transmission of buccal neurons. (a) This diagram illustrates the recording methods used in studying the effects of brief periods of muscle cell contact (0 to 30 min) on release capabilities of buccal neurons in cell culture. SLT muscle fibers were penetrated with microelectrodes or voltage clamped in the whole-cell mode with patch pipets and manipulated into contact with neuronal processes of a cultured buccal neuron. (b) During brief contact of SLT muscle with a neurite of neuron B19, SSPs were recorded in the muscle fiber (left traces). However, ESPs (middle traces) and electrical coupling (right traces) were not detected. (c) During brief contact of SLT muscle fibers with neurites of neuron B5 both SSPs (left traces) and ESPs (middle traces) were detected. Electrical connections did not form between B5 and SLT fibers during brief periods of contact (right trace). (d) Histograms represent the percentage of buccal neuron-SLT muscle fiber brief contacts in which SSPs and ESPs were detected. Vertical scale = 5 mV (b and C, top traces) and 20 mV (b and c, bottom traces). Horizontal scale = 2 see (b and c).

To overcome this problem, another experimental approach was employed to study the effect of SLT muscle cell contact on neuron B19's secretory capacity. Neuron B19 was plated into CM and allowed to initiate neurite extension in cell culture. Several SLT muscle fibers were then plated into coculture with the neuron and the nerve-muscle preparations were maintained under these conditions for 48 hr. The cultures were then incubated for 4 hr in 1-5% hemolymph prior to assay for neurotransmitter release capabilities. An ACh-sensitive neuronal soma was then transferred to the culture dish and recorded from in either current *(n* = 6) or voltage clamp mode *(n = 7)* and manipulated into contact with a neurite of B19 near the site of existing contact with a muscle fiber (Fig. 5a). Such B19 assay neurons are referred to as B19a to distinguish them from preparations in which B19 had extended processes in

culture and was either a test presynaptic neuron or a target cell for synaptic interactions. In 100% of these nerve-muscle preparations *(n = 13* of 13), the spontaneous release of neurotransmitter was detected by the neuronal assay cell, B19a (Fig. 5d). In comparison, spontaneous synaptic events were lower in frequency (see below) and were detected in fewer control preparations *(73.3%; n* = 11 of 15; Fig. 5d) when SLT muscle had not made contact with presynaptic neuron B19.

In addition to spontaneously released neurotransmitter, action potential-evoked release of neurotransmitter was detected by B19a which contacted the neurites or growth cones of neurons B19 which had already existing contact with SLT muscle fibers (4 of *13* preparations; Figs. 5c and 5d). ESPs were never detected in any of the control preparations (0 of 15 cell pairs) without SLT muscle contact. The action potential-evoked release of transmitter observed in experimental nerve-muscle cultures followed presynaptic action potentials in neuron B19 at a constant latency in any given preparation (Fig. 5c). The mean ESC amplitude, measured after *30* min of contact with a voltage clamped assay somata, was *22.1 ± 2.2* pA (mean ± sem; *n = 2* preparations).

These ESCs were the first evidence that neuron B19, under specific culture conditions, can gain the ability to form chemical connections with novel postsynaptic target cells. It was possible to apply pharmacological agents to the medium bathing these neuronal assay preparations to further characterize the neurotransmitter(s) secreted by neuron B19. Bath application of 10^{-5} M tubocurarine reversibly reduced the amplitude of action potential-evoked synaptic potentials detected in neuronal assay somata *(n = 3* of *3;* Fig. 6c). However, slow SSPs persisted in the presence of this cholinergic antagonist. In addition, perfusion of the nerve-muscle culture with 0 mM Ca", 10 mM Mg" medium reversibly blocked the ESPs *(n = 2* of *2;* Fig. 6c).

High fidelity recordings of synaptic interactions were obtained in seven preparations where B19a was voltage clamped with a patch pipet. SSCs detected in these assay somata while contacting neurites of neuron B19 in SLT cocultures were heterogeneous in waveform. The majority of SSCs (77.9% of all SSCs recorded; *n = 7* preparations) detected in these

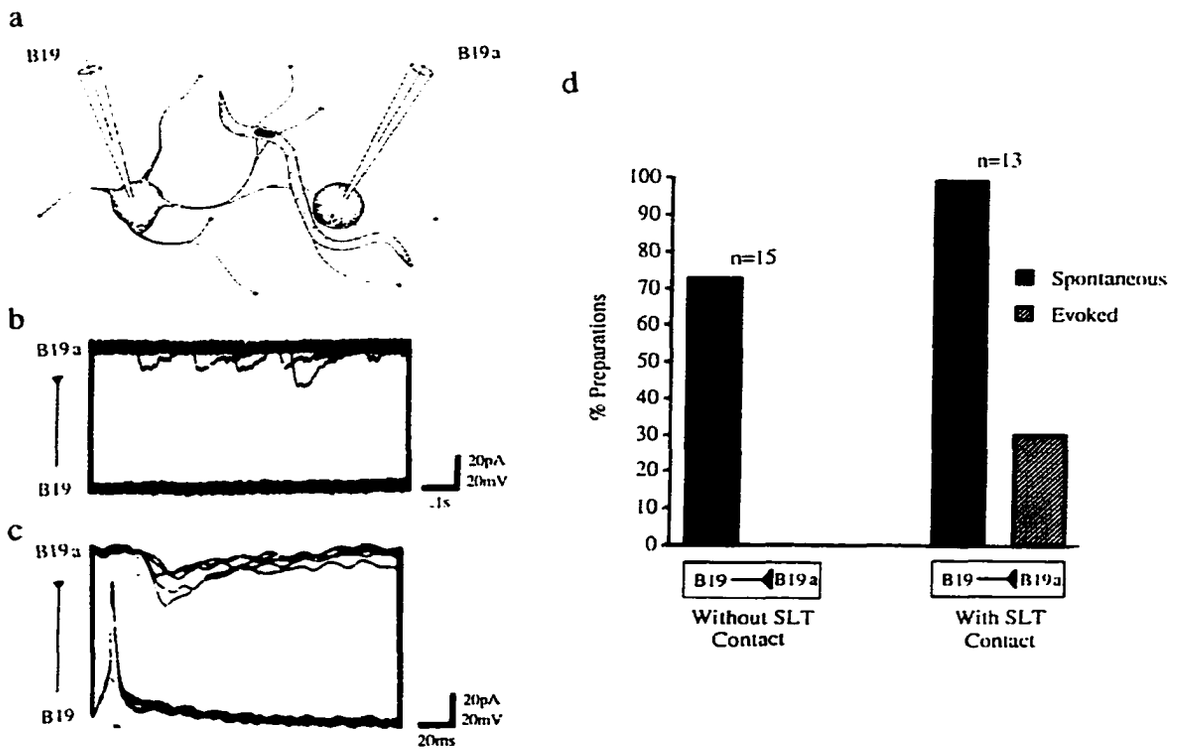

FIG. 5. Effect of sustained muscle cell contact on synaptic transmission of buccal neurons. (a) This diagram illustrates the recording methods used in studying the effects of sustained muscle cell contact (2 days) on neurons for 2 days. Following a 4-hr incubation of the presynaptic neuron B19 in 1% snail hemolymph, ACh-sensitive neuronal somata (1319a) were transferred to the coculture dish, penetrated with microelectrodes or voltage clamped in the whole-cell mode with patch pipets, and manipulated into contact with neuronal processes near the site of muscle contact. (b) Following sustained periods of SLT muscle contact, SSCs were reliably recorded in the neuronal assay cells. Approximately 1 min of continuous, superimposed oscilloscope record is shown. (c) Following sustained contact of B19 with SLT muscle fibers, action potentials in neuron B19 evoked synaptic currents in the novel neuronal assay somata with a constant latency (five superimposed oscilloscope sweeps). (d) In control neuron B19 cultures (without SLT muscle contacts), SSCs, but not ESCs, were detected by assay somata. In experimental cocultures of neuron B19 and SLT fibers, both SSCs (or SSPs) and ESCs (or ESPs) were detected. Histograms represent the percentage of neuron B19-B19 assay cell contacts, in which SSPs and ESPs were detected. Electrical connections did not form between B19 and assay somata in control (without SLT contact) or experimental (with SLT contact) cocultures.transmitter release capabilities of neuron B19. SLT muscle fibers were cultured in contact with processes of buccal

nerve-muscle cultures were fast, transient events (Fig. 6a). These fast SSCs had a time constant of decay of 33.0 + 19.7 ms (mean ± *SD; n = 86* randomly chosen events). The remaining 22.1% of the recorded events were slow, moderate to long-lasting SSCs with decay time constants of 223.5 ± 126.2 ms *(n = 22* random events). Bath perfusion of these nerve-muscle cultures with 10^{-5} M tubocurarine reversibly reduced the amplitude or

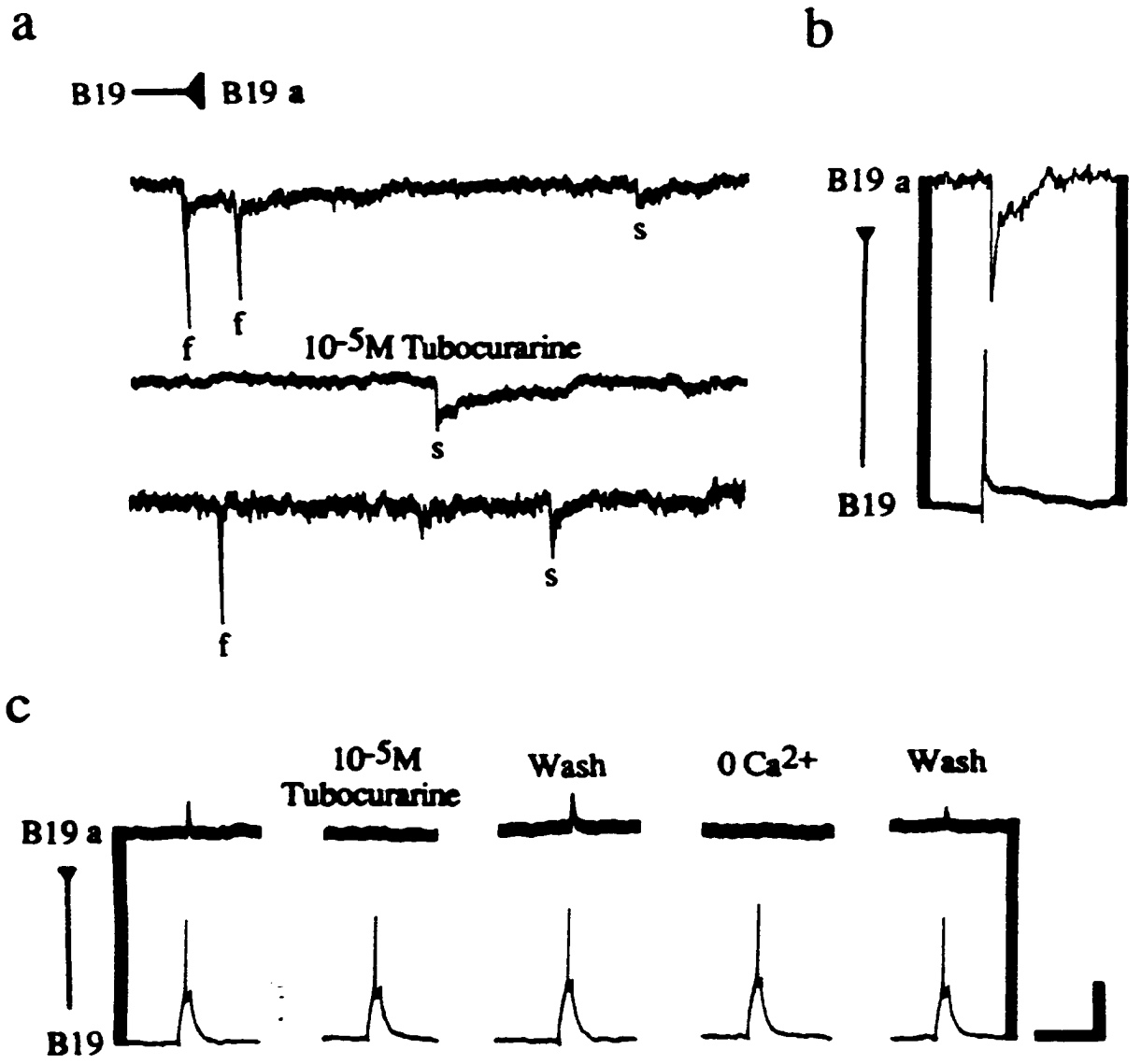

FIG. 6. Neurotransmitter release induced from neuron B19 by SLT muscle contact. (a) SSCs detected in voltage clamped assay somata, following micromanipulation of the novel neuronal target into contact with neurites of a B19 which had already been cocultured for 2 days in contact with SLT muscle fibers, were heterogeneous in waveform. Both fast (f) and slow (s) were recorded. The presence of M tubocurarine reversibly blocked fast SSPs; however, slow SSPs remained. (b) ESC elicited in assay somata by an action potential in presynaptic B19. (c) ESPs elicited by action potentials in the presynaptic B19 were reversibly reduced in amplitude by 10-5** M tubocurarine and 0 mM Ca", 10 mM Mg". Vertical scale = 20 pA (a, all traces; b, top trace), 20 mV (b, bottom trace), 50 mV (c, bottom traces), and 10 mV (c, top traces). Horizontal scale - 2 see (a and c) and 0.4 see (b).

completely blocked the fast *SSCs (n = 7* preparations; Fig. 6a), but did not affect the slow SSCs. These data suggest that two distinct populations of SSCs exist in these preparations.

SLT Muscle Fiber Contact Increases the Rate of Spontaneous Transmitter Release from Neuron B19

Muscle cells have been shown to increase spontaneous ACh release from growth cones of developing *Xenopus*
neurons in cell culture (Xie and Poo, 1986). To better quantify the influence of SLT muscle contact on neuron B19's ability to spontaneously release neurotransmitter, spontaneous events were monitored for both control (without muscle contact) and experimental (with muscle contact) cultures (see Fig. 7 insets) and the average rates of spontaneous release during the first 10 min of contact were calculated.

Three classes of control preparations were used in this experiment and their SSC rates and amplitudes were pooled into one mean value since they failed to be representative of statistically distinct populations. Neurite-bearing B19 was cocultured in contact with novel

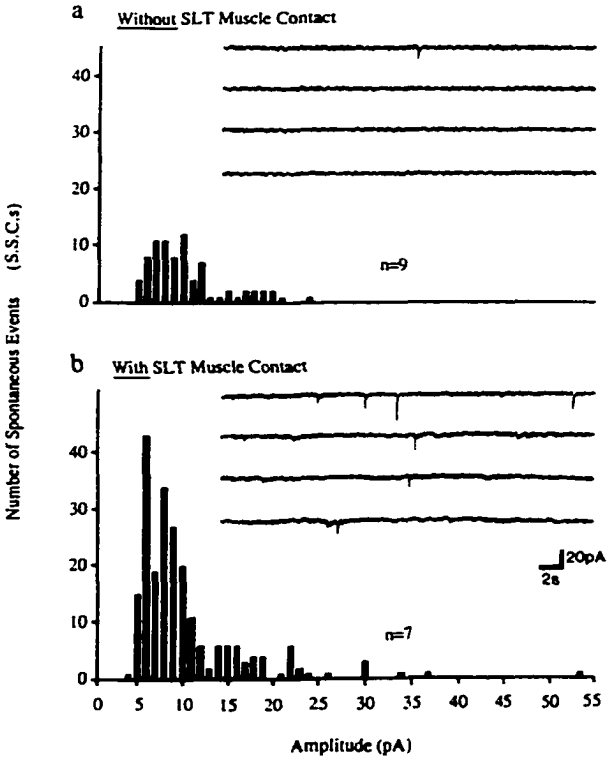

FIG. 7. SLT muscle contact increases the rate of spontaneous synaptic transmission of neuron B19. (a) The distribution of SSC amplitudes recorded from nine control neurons B19 in the absence of SLT muscle fiber contacts during the first 10 min of ACh-sensitive assay cell contact. The mean rate of spontaneous release was 0.9 SSC/min and the mean SSC amplitude was 10.6 pA. The inset illustrates four randomly chosen, 30-sec records from a control assay somata. (b) The distribution of SSC amplitudes recorded from seven experimental neurons B19 in the presence of SLT muscle fiber postsynaptic contact (10 min of assay). The mean rate of spontaneous release was 3.2 SSCs/min and the mean SSC amplitude was 10.2 pA. The inset illustrates four randomly chosen current records.

neuronal targets *(n = 3)*, cocultured in close proximity to, but not contacting, SLT muscle fibers *(n = 3)*, and cultured alone *(n = 3)*.

The rate of SSCs between B19 and neuronal assay somata was 0.9 ± 0.2 SSCs/min (mean + sem; *n = 9)* for control neurons and 3.2 ± 0.7 SSCs/min *(n = 7)* for experimental neurons with existing muscle contacts. These rates of spontaneous release were significantly different ($P < 0.05$; $t = 2.66$; two-tailed t test). As illustrated in Fig. 7, the greater number of spontaneous events in the nerve-muscle cocultures was not associated with an increase in amplitude. The mean SSC amplitude for control cultures was 10.6 ± 1.4 pA (mean± sem; *n* 9) and the mean for experimental cultures was 10.2 0.6 pA *(n = 7)*. These mean amplitudes werenot significantly different ($P > 0.1$; $t = 0.288$; two-tailed t test).

To determine whether contact between neurite-bearing neuron B19 and B19a had effects on neuron B19's secretory ability over and above the effects of muscle contact, the rate and amplitude of SSCs were calculated during 2-min periods for the first 10 min of assay cell contact. In control cells, without previous muscle contact, spontaneous events were rare during the initial 2min of contact (0.2 ± 0.1 SSCs/min; *n = 9)*. Figure 8a illustrates that the rate of SSCs increased some fivefold during the next 6-8 min and plateaued at the significantly greater ($P < 0.02$; $t = 3.16$; two-tailed t test) rateof 1.1 ± 0.3 SSCs/min *(n = 9)*. In contrast, the initial rate of SSCs detected by B19a during the first 2 min of assay cell contact in neuron B19-SLT muscle cocultures was 3.9 ± 1.3 SSCs/min *(n = 7)* and did not change significantly during the 10-min period of contact (Fig.8a). The mean SSC amplitude in control cultures was initially 5.5 0.4 pA and significantly increased ($P< 0.002$; *n = 9*; $t = 4.98$; two-tailed t test) to 11.7 ± 1.9 pA during the first 10 min of assay cell contact (Fig. 8b). The initial mean SSC amplitude of 10.4 ± 1.4 pA, calculated for experimental nerve-muscle cocultures, was also significantly greater ($P < 0.02$; $t = 3.57$; two-tailed t test) than the initial amplitude of control cultures. The amplitude of SSCs detected by B19a in nerve-muscle cocultures did not significantly change throughout the monitoring period (Fig. 8b).

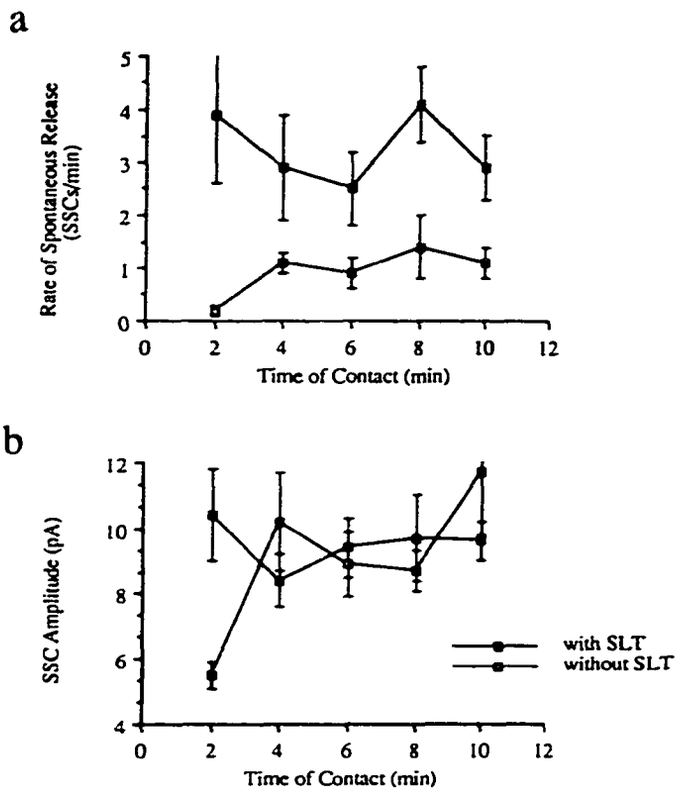

FIG. 8. Effect of brief periods of assay cell contact on the rate of spontaneous release of neurotransmitter. (a) The mean rate of SSCs recorded in assay somata during the first 10 min of contact with neuronal processes of presynaptic B19. Control neurons B19 (open boxes; $n = 9$), cultured without SLT muscle fiber contact, had low rates of spontaneous release during the first 2 min of assay cell contact. Over the next 8 min this rate gradually increased to a plateau value near 1 SSC/min. Experimental neurons B19 (closed boxes; $n = 7$), cocultured for 2 days with SLT muscle fiber contact, had a significantly higher (see text) rate of spontaneous release immediately following assay cell contact. This rate remained relatively constant throughout the next 8 min and significantly higher than the spontaneous release rate of control neurons. (b) The mean amplitudes of SSCs recorded in assay somata during the first 10 min of contact with neuronal processes of presynaptic B19. SSCs of control neurons B19 (open boxes; $n = 9$) had amplitudes that were initially small and gradually increased in size over the first 10 min of contact. The amplitudes of SSCs of experimental B19s in nerve-muscle cocultures (closed boxes; $n = 7$) were significantly larger than those of control SSCs (see text) at the onset of contact. The amplitude of SSCs in experimental cultures did not increase during the first 10 min of contact. Vertical bars represent ±SEM.

DISCUSSION

B19, isolated from the nervous system of *Helisoma* and plated into cell culture, forms its appropriate synaptic connection (i.e., connection normally found *in* vivo) (Zoran *et al.*, 1989) when paired with SLT muscle fibers (Fig. 2). By contrast, when neuron B19 is paired in culture with novel target cells, it does not form the presynaptic element of novel chemical

connections (Table 1; also Haydon and Zoran, 1989). *In vitro* studies of synaptogenesis in *Aplysia* and leech have also demonstrated that specific neurons form a restricted set of appropriate synaptic connections (Camardo *et al.*, 1983; Arechiga *et al.*, 1986; Vyklicky and Nicholls, 1988). While the present study demonstrates that neuron B19 is selective in synaptogenesis, it does not determine whether this selectivity is tissue-specific (i.e., B19 forms chemical connections with any muscle type but not other neurons) or cell-specific (i.e., B19 forms connections only with SLT muscle fibers). Future experiments utilizing novel muscle targets in coculture with B19 will further define the selective nature of this neuron's synaptogenic capacity.

In contrast to neuron B19, neuron B5 of *Helisoma* forms novel chemical connections with every ACh-sensitive target (neuron or muscle) it has been paired with in cell culture (Table 1; Haydon and Kater, 1988; Haydon and Zoran, 1989). In addition, certain identified neurons of Aplysia and leech are also indiscriminate in synaptogenesis and reliably form novel chemical connections (Schacher *et* al., 1985; Arechiga *et* al., 1986). Therefore, these different presynaptic neurons exhibit intrinsic differences in synaptogenic capabilities.

Given that differences exist with regard to synaptogenic capability, there remains the question of what subcellular mechanisms account for these distinctions. Neuron B5, which is indiscriminate in synaptogenesis, is able to release ACh almost immediately on contact (within 3 sec) with novel ACh-sensitive neuronal targets micromanipulated into contact with its neurites and growth cones (Haydon and Zoran, 1989). This rapid onset of functional synaptic transmission suggests that the neurites of synaptogenically unconstrained neurons might acquire neurosecretory properties prior to target contact. Several *in vitro* studies have shown that neuronal growth cones can contain sufficient transmitter and efficient mechanisms of excitation-secretion coupling in the absence of target contact (Hume *et* al., 1983; Young and Poo, 1983; Sun and Poo, 1987). Functional synaptic transmission can occur within the first minutes of cell contact between embryonic *Xenopus* spinal neurons growing in culture and micromanipulated muscle cells (Sun and Poo, 1987).

When novel neuronal or appropriate SLT muscle targets are manipulated into contact with the processes of neuron B19, the presence of action potential -evoked synaptic events is not detected during 30 min of target cell contact (Figs. 4 and 5; also Haydon and Zoran,

1989). Only after long periods of muscle-specific contact did B19 gain the ability to evoke the release of neurotransmitter (Figs. 2 and 5). Thus, unlike neuron B5, neuron B19 requires long periods of cell-specific contact to initiate the final developmental steps involved in the conversion of its presynaptic terminal into a functionally transmitting secretory state.

Micromanipulation experiments using ACh-sensitive assay cells demonstrated that spontaneous release of neurotransmitter, in contrast to evoked transmitter release, could be detected from B19 after brief periods of novel and appropriate target cell contact (Figs. 4 and 5). This observation suggests that B19 possesses a releasable transmitter pool and efficient exocytotic machinery before target contact, but it requires muscle-specific influences to induce the presynaptic acquisition of a functional excitation-secretion coupling mechanism. Since Cohan *et* al. (1987) have provided evidence for the presence of calcium channels in neurites and growth cones of neuron B19 in the absence of target contact, a likely site for the muscle-specific induction of excitation-secretion coupling is at the level of the calciumdetector of the secretory apparatus. Perhaps contact with SLT muscle induces the synthesis of a vesicle-specific protein (Baumert *et* al., 1989; Sudhof *et* al., 1989) which is required for calcium-dependent exocytosis. The requirement of long periods of muscle-specific contact supports such a protein synthesis hypothesis. However, the specific component(s) of the excitation-secretion coupling mechanism missing in the nerve terminal of B19 prior to target contact and the instrument of its acquisition during synaptogenesis remain to be elucidated.

Functional chemical connections were formed in a relatively small percentage of B19-SLT (17.1%; $n = 35$) and B5-SLT preparations (21.4%; $n = 14$), as compared to 80% of neuronal cell pairs possessing functional neurotransmission (see Table 1). Similarly, in muscle fiber micromanipulation experiments, spontaneous release of ACh was detected in only 33.3% of B19-SLT contacts and 28.6% of B5-SLT contacts. This paucity of nervemuscle connections may be the result of problems associated with the contractile nature of the muscle fibers in culture (i.e., many fibers that have formed connections may be lost during irreversible contractions). Alternatively, the possibility exists that the percentage of functionally transmitting neurons may be dependent on the duration of contact with the muscle targets. In addition, the low percentage of nerve-muscle connections in culture may

reflect a variable reduction in ACh-sensitivity of SLT muscle fibers or digestion of muscle surface coat (e.g., basal lamina) during enzymatic dissociation. Since spontaneous release of ACh was detected in 100% of B19-B19a preparations (with SLT muscle contacts; Fig. 5), it is possible that only a subset of all SLT muscle fibers are capable of becoming a postsynaptic cell.

In addition to inducing the capacity for action potential-evoked synaptic transmission, contact with SLT muscle fibers enhanced the spontaneous rate of ACh release from the processes of B19 as detected by micromanipulated assay cells (Fig. 8). Increases in spontaneous release of neurotransmitter (Xie and Poo, 1986; Evers *et al.,* 1989) and the efficacy of action potentialevoked release of neurotransmitter during cell-cell interactions (Kidokoro and Yeh, 1982; Chow and Poo, 1985; Role *et al.,* 1987; Haydon and Zoran, 1989; Evers *et al.,* 1989) have been reported for several synapses formed in cell culture. Similar increases in synaptic efficacy have been documented for many developing synapses in vivo (Dennis, 1981). The mechanisms underlying these contact-dependent effects are likely to be diverse. Evers *et al.* (1989) have hypothesized that such postcontact increases in synaptic efficacy at *Xenopus* neuromuscular synapses might result from the bonding and localization (zippering) of surface molecules associated with the nerve and muscle membranes. The directed axonal transport of vesicles toward regions of synaptic contact have been reported in Aplysia cultured neurons (Goldberg and Schacher, 1987). Much like the postsynaptic aggregation of ACh-receptors which occurs during neuromuscular synaptogenesis (Anderson *et* at, 1977; Frank and Fischbach, 1979), contact-dependent localization of presynaptic calcium channels has been demonstrated during synapse formation in hippocampal neuronal cell cultures (Jones *et* at, 1989). An additional possibility is that the increased rate of spontaneous ACh release is due to an increase in the probability of vesicle fusion resulting from the newly acquired ability to detect internal calcium.

It should be noted that assay for transmitter release with neuronal somata requires that B19a contact the presynaptic neuron at a site adjacent to the nerve muscle synaptic site. Chow and Poo (1985) have suggested that release capabilities from noncontact sites might be reduced due to down-regulation of the machinery for action potential-evoked release in these

regions. This might in part explain the low percentage of nerve-muscle preparations in which evoked release of neurotransmitter was detected by the B19 assay cell.

The rate of spontaneous release of ACh from neuron B19 is relatively high (approximately 1 to 5 SSCs/min) in most preparations during the first minutes of contact with the novel neuronal assay cell (Figs. 5b and 8a). In contrast, spontaneous release events are rare in chronic cocultures of B19 and novel neuronal targets. This observation raises the possibility that "negative" recognition may occur between novel partners such that after periods of greater than 30 min of contact, synapse elimination may occur. Indeed, in experiments performed in vivo, Haydon and Kater (1988) showed that regenerating neuron B5 forms transient novel chemical connections following perturbation of the adult nervous system. It is perhaps not coincidental that, although B19-B19 cell pairs form their appropriate electrical connections (Kater, 1974) following prolonged periods of contact in cell culture (Table 1; also Hadley and Kater, 1983), electrical coupling is not detected during the first 30 min of B19-B19a contacts (Fig. 5). Spontaneous release of neurotransmitter between these novel partners, however, is detected at this early time of contact. Perhaps the emergence of electrical coupling promotes the subsequent elimination of neuron B19's secretory capacity.

In conclusion, these results demonstrate that following contact with appropriate SLT muscle fibers neuron B19 gains the ability to couple excitation with secretion and, consequently, is capable of forming the presynaptic element of functional synaptic connections. Taken together with previous studies (Haydon and Kater ' 1988; Haydon and Zoran, 1989), these results suggest that synaptogenically unconstrained neuron B5 requires little target-derived influence or presynaptic change to form initial functional connections. However, the sustained time of SLT muscle contact that is required to gain functional secretory capabilities by synaptogenically restricted neuron B19 suggests that both appropriate target contact and presynaptic modifications (i.e., transcription, translation, and/or phosphorylation) might be involved in the transformation of this neuron's growth cone into a functional nerve terminal.

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REFERENCES

AMBRON, R. T., DEN, H., and SCHACHER, S. (1985). Synaptogenesis by single identified neurons in *vitro*.Contribution of rapidly transported and newly synthesized proteins. J. *Neurosci. 5,* 2857-2865.

ANDERSON, M. J., COHEN, M. W., and ZORYCHTA, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. J. Physiol. 268, 731-756.

ARECHIGA, H., CHIQUET, M., KUFFLER, D. P., and NICHOLLS, J. G. (1986). Formation of specific connections in culture by identified leech neurones containing serotonin, acetylcholine, and peptide transmitters. J. Exp. Biol. 126,15-31.

BAUMERT, M., MAYCOX, P. R., NAVONE, F., DE CAMILLI, P., and JAHN, R. (1989). Synaptobrevin: An integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO* J. 8, 379-384.

BODMER, R., DAGAN, D., and LEVITAN, 1. B, (1984). Chemical and electrotonic connections between Aplysia neurons in primary culture. J. *Neurosci. 4,* 228-233.

BUCHANAN, J., SUN, Y-a., and *Poo,* M-m. (1989). Studies of nervemuscle interactions in *Xenopus* cell culture: Fine structure of early functional contacts. J. *Neurosci. 9,* 1540-1554.

CAMARDO, J. E., PROSHANSKY, E., and SCHACHER, S. (1983). Identified *Aplysia* neurons form specific chemical synapses in culture. J. Neurosci. 3, 2614-2620.

CHIQUET, M., and NICHOLLS, J. G. (1987). Neurite outgrowth and synapse formation by identified leech neurones in culture. J. Exp. *Biol.* 132,191-206.

CHOW, L, and *Poo,* M-m. (1985). Release of acetylcholine from embryonic neurons upon contact with muscle cell. J. *Neurosci. 5,* 1076-1082.

COHAN, C. S., CONNOR, J. A., and KATER, S. B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J. *Neurosci. 7,* 3588-3599.

DENNIS, M. J. (1981). Development of the neuromuscular junction: Inductive interactions between cells. *Annu. Rev. Neurosci. 4,* 43-68.

EVERS, J., LASER, M., SUN, Y-a., XIE, Z-p., and Poo, M-m. (1989). Studies of nerve-muscle interactions in *Xenopus* cell culture: Analysis of early synaptic currents. J. *Neurosci. 9,* 1523-1539.

FRANK, E., and FISCHBACH, G. D. (1979). Early events in neuromuscular synapse formation *in vitro*. J. *Cell Biol.* 83, 143-158.

FUCHS, P. A., NICHOLLS, J. G., and READY, D. F. (1981). Membrane properties and selective connexions of identified leech neurones in culture. J. Physiol. 316, 203-223.

GOLDBERG, D. J., and SCHACHER, S. (1987). Differential growth of the branches of a regenerating bifurcate axon is associated with differential axonal transport of organelles. *Dev. Biol.* 124, 35-40.

GREGA, D. S., and KATER, S. B. (1987). Reinitiation of outgrowth from dormant neurons in cell culture. Soc. *Neurosci. Abstr.* 13, 167.

HADLEY, R. D., BODNAR, D. A., and KATER, S. B. (1985). Formation of electrical synapses between isolated, cell cultured *Helisoma* neurons requires mutual neurite elongation. *J. Neurosci.* 5, 3145-3153.

HADLEY, R. D., and KATER, S. B. (1983). Competence to form electrical connections is restricted to growing neurites in the snail, *Helisoma. J. Neurosci.* 3, 924-932.

HADLEY, R. D., KATER, S. B., and COHAN, C. S. (1983). Electrical synapse formation depends on interaction of mutually growing neurites. *Science* 221, 466-468.

HAYDON, P. G. (1988). Chemical synapses form between isolated neuronal somata in the absence of neurite extension. *J. Neurosci.* 8, 1032-1038.

HAYDON, P. G., COHAN, C. S., McCOBB, D. P., MILLER, H. R., and KATER, S. B. (1985). Neuron specific growth cone properties as seen in identified neurons of *Helisoma. J. Neurosci. Res.* 13, 285-300.

HAYDON, P. G., and KATER, S. B. (1988). The differential regulation of formation of electrical and chemical connections in *Helisoma. J. Neurobiol.* 19, 636-655.

HAYDON, P. G., and MAN-SON-HING, H. J. (1988). Low- and high-voltage-activated calcium currents: Their relationship to the site of neurotransmitter release in an identified neuron of *Helisoma. Neuron* 1, 919-927.

HAYDON, P. G., and ZORAN, M. J. (1989). Formation and modulation of chemical connections: Evoked acetylcholine release from growth cones and neurites of specific identified neurons. *Neuron* 2, 1483-1490.

HAYDON, P. G., and ZORAN, M. J. (1990). Chemical synapses in culture. *In* "Cellular and Molecular Neurobiology: A Practical Approach" (H. Wheal and J. Chad, Eds.). IRL Press, Oxford.

HAYDON, P. G., ZORAN, M. J., Man-Son-Hing, H. J., SIEVERS, E. M., and DOYLE, R. T. (1990). A relation between specificity and the acquisition of presynaptic properties. *J. Physiol. (Paris),* in press.

HUME, R. I., ROLE, L. W., and FISCHBACH, G. D. (1983). Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature (London)* 305, 632-634.

JONES, 0. T., KUNZE, D. L., and ANGELIDEs, K. J. (1989). Localization and mobility of w-conotoxin-sensitive Ca" channels in hippocampal CAI neurons. *Science* 244, 1189-1192.

KATER, S. B. (1974). Feeding in *Helisoma trivolvis:* The morphological and physiological bases of a fixed action pattern. *Amer.* Zool. 14, 1017-1036.

KIDOKORO, Y., and YEH, E. (1982). Initial synaptic transmission at the growth cone in *Xenopus* nerve-muscle cultures. *Proc. Natl. Acad.* Sci. USA 79,6727-6731.

LIN, S. S., and LEVITAN, 1. B. (1987). Concanavalin A alters synaptic specificity between cultured *Aplysia* neurons. *Science* 237, 648-650.

READY, D. F., and NICHOLLS, J. G. (1979). Identified neurones isolated from leech CNS make selective connections in culture. *Nature (London)* 281, 67-69.

ROLE, L. W., ROUFA, D. G., and FISCHBACH, G. D. (1987). The distribution of acetylcholine receptor clusters and of sites of transmitter release along chick ciliary ganglion neurite-myotube contacts in culture. *J. Cell* Biol. 104, 371-379.

SCHACHER, S., RAYPORT, S. G., and AMBRON, R. T. (1985). Giant *Aplysia* neuron R2 reliably forms strong chemical connections *in vitro. J. Neurosci.* 5, 2851-2856.

SUDHOF, T. C., BAUMERT, M., PERIN, M. S., and JAHN, R. (1989). A synaptic vesicle membrane protein is conserved from mammals to *Drosophila. Neuron* 2,1475-1481.

SUN, Y-a., and Poo, M-m. (1987). Evoked release of acetylcholine from the growing embryonic neuron. *Proc. Natl. Acad. Sci. USA* 84, 2540-2544.

TAKAHASHI, T., NAKAJIMA, Y., HIROSAWA, K., and ONODERA, K. (1987). Structure and physiology of developing neuromuscular synapses in culture. *J. Neurosci.* 7, 473-481.

VYKLICKY, L., and NICHOLLS, J. G. (1988). Specificity of connections formed by nociceptive cells of the leech in tissue culture. *J. Exp. Biol.* 134,17-26.

WONG, R. G., HADLEY, R. D., KATER, S. B., and HAUSER, G. C. (1981). Neurite outgrowth in molluscan organ and cell cultures: The role of conditioning factor(s). *J Neurosci.* 1, 1008-1021.

XIE, Z-p., and Poo, M-m. (1986). Initial events in the formation of neuromuscular synapse: Rapid induction of acetylcholine release from embryonic neurons. *Proc. Natl. Acad. Sci. USA* 83, 7068-7073.

YOUNG, S. H., and Poo, M-m. (1983). Spontaneous release of neurotransmitter from growth cones of embryonic neurones. *Nature (London)* 305, 634-637.

ZORAN, M. J., HAYDON, P. G., and MATTHEWS, P. J. (1989). Aminergic and peptidergic modulation of an identified motor pathway in the gastropod mollusc, *Helisoma. J. Exp. Biol.* 142, 225-243.

APPENDIX B. TARGET CONTACT REGULATES THE CALCIUM RESPONSIVENESS OF THE SECRETORY MACHINERY DURING SYNAPTOGENESIS

Target Contact Regulates the Calcium Responsiveness of the Secretory Machinery during Synaptogenesis

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Summary

Neuron B19 of Helisoma is selective in synaptogenesis. Presynaptic mechanisms underlying this selectivity were tested. Acetylcholine-sensitive assay cells were micromanipulated into contact with B19 somata to assess its secretory state. Prior to appropriate muscle target contact, spontaneous synaptic currents were detected; however, action potential-evoked release of neurotransmitter was detected only following hours of muscle contact. Photolysis of a calcium cage, DM-nitrophen, accelerated the frequency of synaptic currents in muscle-contacted, but not novel neuron-contacted, B19 somata. These studies demonstrate that contact with appropriate target muscle enhances the responsiveness of this neuron's secretory machinery to internal calcium levels, thereby imparting the presynaptic cell with the ability to couple action potentials with neurotransmitter release.

Introduction

Sperry's classic experiments on the regeneration of retinal axons suggested that specificity of neural connections may be achieved through processes of target recognition based on chemoaffinities (Sperry, 1963). Studies of synaptogenesis in recent years have argued that specific recognition of surface labels might be less important than once thought in the establishment of appropriate synaptic connections (Easter et al., 1985). Our previous studies have examined the specificity of synaptogenesis of two putative cholinergic neurons and have examined the role that target recognition plays in formation of their synaptic connections (Haydon and Zoran, 1989; Zoran et al., 1990). Neuron B19, isolated from the buccal ganglion of Helisoma and plated into cell culture, is restricted in synaptogenesis and never forms the presynaptic element of novel chemical connections (Haydon and Kater, 1988; Haydon and Zoran, 1989). Rather, neuron B19 requires recognition of an appropriate target to gain the ability to release neurotransmitter in response to action potentials and become an adequate presynaptic element (Zoran et.al., 1990). In contrast to neuron B19,

cholinergic neuron B5 is nonselective in synaptogenesis and reliably forms novel chemical connections (Haydon and Kater, 1988; Haydon and Zoran, 1989). These experiments, in the case of neuron B19, support the idea that target recognition is important in synapse formation; however, in the case of neuron B5, chemical recognition of its synaptic targets seems much less likely.

Although cellular interactions are important in synapse formation (Chow and Poo, 1985; Sun and Poo, 1987; Takahashi et al., 1987; Evers et al., 1989), little is known about presynaptic mechanisms involved in the synaptogenic process. Recent studies have shown that the secretory machinery of neuron B19 gains the ability to support synaptic transmission following sustained periods of muscle-specific contact (Zoran et al., 1990). Prior to muscle contact, action potentials are unable to stimulate neurotransmitter release. Minutes after muscle contact, spontaneous synaptic currents are detected. However, several hours of B19-muscle contact are required before action potentials in neuron B19 evoke synaptic transmission (Zoran et al., 1990). Since spontaneous synaptic transmission is detected upon muscle contact, the secretory machinery is initially present in neuron B19, but a component of the synaptic terminal crucial for excitation-secretion coupling is absent. We have hypothesized that the induction of a functional synaptic terminal could be due to at least two target-dependent mechanisms: contact induces the appearence of presynaptic calcium channels in neuron B19, or contact changes the state of the secretory machinery such that it becomes responsive to calcium, allowing action potentials to evoke neurotransmitter release.

To test these hypotheses adequately, it was necessary to have direct electrophysiological access to the site of neurotransmitter release (i.e., the nerve terminal). For this reason, we used a cell culture protocol for obtaining giant somatic synapses (Haydon, 1988) in which both the ionic conductances and the status of the secretory machinery of neuron B19 could be examined in the presence of appropriate target contacts (i.e., cells it normally synapses with in the intact nervous system) and novel target contacts (i.e., cells it does not synapse with in the unperturbed nervous system). Using voltage- and calcium-clamp techniques, this study demonstrates that the transformation of neuron B19 into a functional presynaptic state following appropriate muscle contact involves a change in the responsiveness of its secretory apparatus to free intracellular calcium.

Results

B19 somata were isolated from the adult nervous system of Helisoma and were cultured for 24 hr on a nonadhesive substrate that discourages neurite outgrowth. Spherical neurons were cultured for an additional 48 hr in contact with several supralateral radular tensor (SLT) muscle fibers (Figure 1A). During the 48 hr of B19-SLT muscle fiber coculture, neuron B19 maintained its spherical geometry and did not extend neuritic processes over the muscle fibers, as demonstrated by Lucifer yellow fills of the presynaptic neuron B19 (Figure 1B).

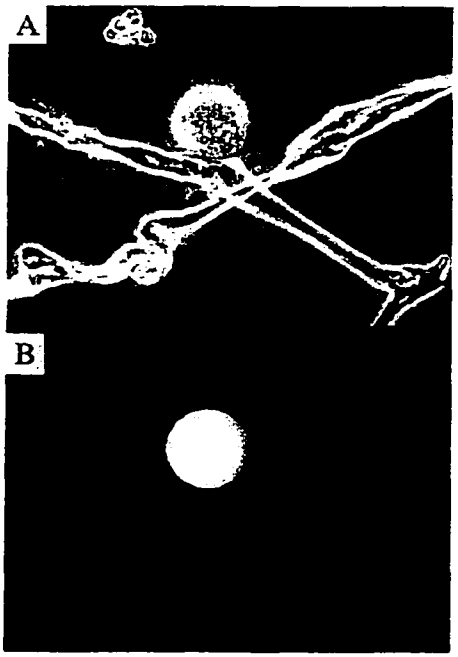

Figure 1. Helisoma Neuron B19-SLT Muscle Culture
(A) The spherical cell body of a neuron B19 plated in contact with several SLT muscle fibers in cell culture. (B) After 48 hr of culture, neuron B19 was iontophoretically filled with the fluorescent dye Lucifer yellow. Note that the neuron has maintained a spherical geometry, and neurites have not extended over the adjacent muscle fibers. The diameter of neuron B19 is 50 μm.

Previous quantitative studies of the B19-SLT neuromuscular synapse formation were hampered by irreversible damage caused to muscle fibers during short-lived intracellular recordings (Zoran et al., 1990). Therefore, we modified a myoball technique first utilized by Chow and Poo (1985). In this technique an acetylcholine (ACh)-sensitive assay cell was manipulated into contact with the presynaptic neuron close to the site of neuromuscular contact to "sniff" ACh released at or near the neuromuscular synapse (Zoran et al., 1990). We used the same approach to detect evoked and spontaneous synaptic currents (ESCs and SSCs) at giant B19 somatic synapses in this study.

B19 somata, like neurite-bearing neuron B19, gain secretory capabilities following contact with SILT muscle fibers. An ACh-sensitive assay cell (see Experimental Procedures for culture protocol) was manipulated into contact with the presynaptic neuron for 30 min. The presynaptic cell was then penetrated with a conventional intracellular microelectrode for current clamp, and the assay cell was voltage-clamped in the whole-cell mode for the detection of synaptic transmission. Action potentials were evoked in the presynaptic cell by injection of depolarizing current, and the simultaneous current responses of the assay cell were monitored. As shown in Figure 2A, action potential evoked release of neurotransmitter from SLT-contacted neurons was detected by the assay cell as inward currents. ESCs followed the presynaptic spikes at a constant latency in a given preparation. Action potential-evoked release was found in 75% of the SLT-contacted preparations (n = 8; Figure 2B). ESCs were never detected in control preparations in which neuron B19 made contact with novel target neurons (n = 10; Figures 2A and 2B). SSCs were detected, however, in 100% of SLT-contacted and control preparations (Figures 2A and 2B). The rate of spontaneous release was 2.3 $\pm$ 2.2 SSCs per min (mean + SID; n = 8) for SILT-contacted neuron B19 preparations and 1.1 $\pm$ 1.0 SSCs per min (n = 12) for neuron-contacted controls. The mean rates of spontaneous release were not statistically different. These results with spherical neurons are consistent with those previously attained when neurite-bearing neuron B19 made contact with SILT fibers on its processes (Zoran et al., 1990). Thus, the spherical presynaptic cell parallels the synaptogenic properties of neurite-bearing cells.

Action potentials in neuronal processes of neuron B19 elicit rapid increases in intracellular calcium levels, presumably via voltage-dependent calcium channels (Cohan et al., 1987). Since contact with appropriate muscle targets causes a change in spherical B19 somata into a functional presynaptic state (Figure 2), we determined whether SILT contact changes the types or density of calcium currents in the B19 soma. To examine this possibility, spherical B19 somata were cultured for 48 hr under three conditions: alone, in contact with a novel neuronal target, or in contact with SLT muscle fiber targets. Neurons were voltage-clamped in the whole-cell configuration under pharmacological and ionic conditions that allow the detection of macroscopic calcium currents (see Experimental

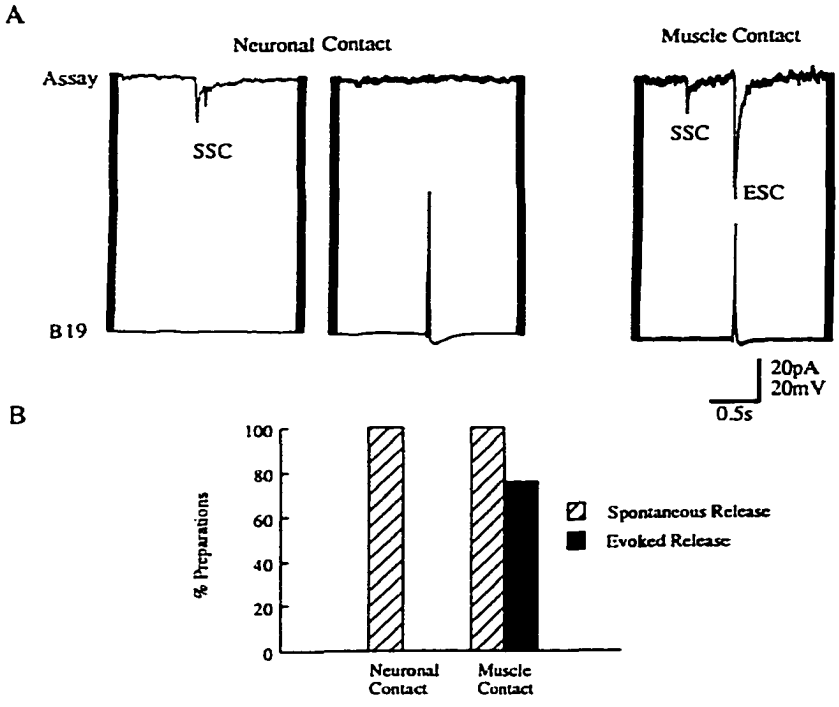

Figure 2. Target-Dependent Induction of Action Potential-Evoked Release of Neurotransmitter from B19 Somata (A) An action potential evoked in neuron B19, following 48 hr of contact with a novel neuronal target, fails to elicit a synaptic current in an ACh-sensitive assay cell. However, an action potential in a muscle (SLT)contacted neuron B19 does evoke a synaptic current (ESQ. (B) Neuronal somata of neuron B19 spontaneously released neurotransmitter, as detected by SSCs in the assay cell, in all preparations, whether contacted by novel neuronal (n 10) or appropriate muscle (n = 8) target. Action potential-evoked release was seen only in muscle-contacted cells. Histograms represent the percentage of preparations (cells) in which SSCs and ESCs were detected.

Procedures for ionic solutions; Haydon and Man-Son-Hing, 1988). Somata were depolarized from holding potentials of -60 mV to command potentials of -20to +50 mV. These experiments showed that calcium currents in each group were of the high-voltage-activated type (Figure 3A; Haydon and Man-Son-Hing, 1988) and that calcium current densities were not significantly different between groups (Figure 3B).

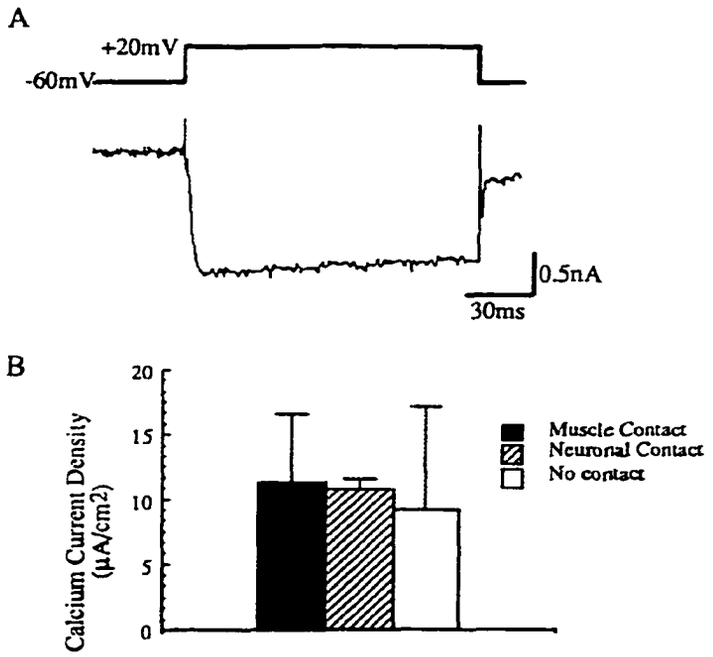

Figure3. High-Voltage-Activated Calcium Current of a Spherical Neuron B19 (A) The macroscopic calcium current of a B19 soma is shown following 48 hr of contact with a novel neuronal target cell (another neuron B19). (B) Histograms demonstrate the density of high-voltage-activated calcium current in B19 somata following 48 hr of contact with appropriate SLT muscle (n = 5), contact with novel neurons (n = 5), and no contact (n = 4). Bars represent means ± SD.

Since neuron B19 possesses similar voltage-activated calcium current densities with and without appropriate target muscle contact, the dramatic transformation of this presynaptic neuron into a secretory state is not likely to be due to a change in the status of calcium current in the secretory membrane. We cannot, however, exclude the possibility that a minor population of novel channels are induced to appear at the site of contact.

To determine whether the calcium responsiveness of the secretory apparatus changes following target contact, the level of cytoplasmic calcium was manipulated with a photolabile calcium cage, DM-nitrophen (Kaplan and Ellis-Davies, 1988). DM-nitrophen has previously been used to elicit calcium-evoked transmitter release at the squid giant synapse (Delaney and Zucker, 1990). In the study presented here, neuronal B19 somata were cultured for 48 hr either in the presence of appropriate muscle fiber contact or in the presence of novel

neuronal contact. At this time, an ACh-sensitive assay cell was manipulated into contact with the presynaptic neuron for 30 min prior to electrophysiological recording. Presynaptic neuronal somata were then penetrated with a micropipette containing 75 mM DM-nitrophen, 21.6 mM CaC12, and 1 mM fluo-3 (see Experimental Procedures for complete procedures). Fluo-3, a calcium-sensitive fluorescent dye, was added to the internal injection solution to provide a mechanism for monitoring the resting and elevated levels of presynaptic intracellular calcium throughout the experiment. The assay cell was then voltage-clamped in the whole-cell configuration, and the preparation was allowed to stabilize for 10 min, during which time the rate of SSCs was monitored. The presynaptic neuron was then pressure-injected with the 30% calcium-loaded nitrophen solution. After reaching a new baseline rate of SSCs, calcium was released from the cage by a 3 s exposure to UV light and neurotransmitter release, detected by the assay cell, was recorded.

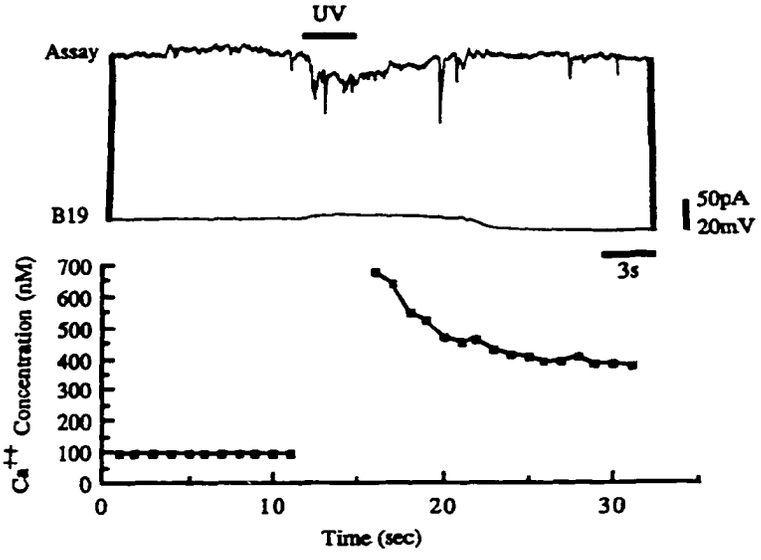

Figure 4. Photolysis of Injected DM-Nitrophen Elevates Intracellular Calcium Levels and Elicits an Increase in the Rate of Transmitter Released from the Soma of an SLT Muscle-Contacted Neuron B19
Simultaneous recordings of presynaptic voltage (lower trace) and assay cell current (upper trace) before, during, and after epi-illu mi nation with UV light are shown. Intracellular levels of free calcium were also simultaneously estimated using fluorescent emission of the calcium-sensitive dye fluo-3 (bottom graph). Note that following a 3 s UV illumination, inward currents were detected in the voltage-clamped assay cell and an initially large, transient increase in intracellular free calcium was seen. An elevated level of free internal calcium was then sustained.

Photolysis of DM-nitrophen elevated intracellular calcium and triggered the release of neurotransmitter from neuronal B19 somata that had contacted SILT muscle fibers for 48 hr

(Figure 4; Figure 5A). Upon photolysis, the free cytosolic calcium level was raised in the presynaptic neuron, as estimated from fluo-3 emissions recorded before and after the UV flash (Figure 4). Filters for UV photolysis and for fluo-3 excitation were manually changed. Thus, we were unable to determine the calcium level during the period of photolysis. Rather, we use fluo-3 to confirm that photolysis had effectively raised intracellular calcium levels. Elevated calcium levels elicited release of transmitter, as indicated by the accelerated frequency of synaptic currents (SCs) recorded in the assay cell (n = 5). Presynaptic resting membrane potential was only modestly affected by photolysis. To control for the effects of photo-products, calcium-free DM-nitrophen was injected into presynaptic neuron B19. Photolysis of calcium-free DM-nitrophen had no effect on calcium levels in neuron B19 and did not evoke neurosecretion (n = 3; Figure 5C). Thus, the release of neurotransmitter following photolysis of calciumloaded DM-nitrophen is calcium dependent.

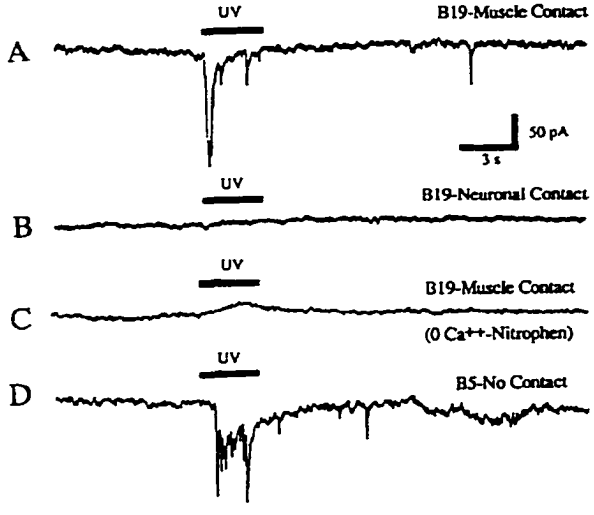

Figure 5. Target Dependence of Photolysis-Evoked Transmitter Release from DM-Nitrophen-Injected Spherical Neuronal Somata. Photolysis of 30% calcium-loaded DM-nitrophen elicited neurotransmitter release from muscle-contacted (A), but not neuroncontacted, B19 somata (B). In these examples, photolysis elevated internal calcium, as estimated by fluo-3 fluorescence, from 105 nM (A) and 115 nM (B) before UV flash to 229 nM (A) and 436 nM after flash. Transmitter release was not detected from musclecontacted neuron B19 injected with calcium-free DM-nitrophen (C). UV irradiation of calcium-free DM-nitrophen had no effect on intracellular calcium levels. Photolysis of30% calcium-loaded DM-nitrophen also elicited transmitter release from neuron B5 cultured for 48 hr without any target contact (D). Photolysis increased internal calcium of this neuron B5 from 92 nM before UV flash to 327 nM after flash. All traces represent whole-cell current recordings from voltage-clamped assay cells that had contacted the neuronal somata for 30 min prior to the recording.

The mean rates of SCs were determined for the 10 s period before onset of the UV exposure and for the 10 s period following the onset of exposure (3 a during photolysis and 7 s immediately following). The rate of release was elevated in 5 of 5 SLT muscle-contacted neuron B19 preparations from 0.20 $\pm$0.13 SCs per s (mean $\pm$SID) before photolysis to 0.90 $\pm$0.46 SCs per s during the first 10 s following photolysis. In contrast, photolysis of 30% calcium-loaded DM-nitrophen never increased the rate of release in control preparations of neuron B19 with novel neuronal contacts (0 of 5 preparations; Figure 5B). Mean release rates were 0.02 $\pm$ 0.04 SCs per s before photolysis and 0.02 $\pm$0.04 SCs per s after photolysis. Although photolysis failed to evoke secretion, a rise in calcium level was achieved in each of these preparations.

Nitrophen photolysis experiments demonstrated that transmitter release could be evoked from neuron B19, a neuron restricted in its synaptogenic capabilities, only when calcium levels were elevated and the cell had previously contacted an appropriate muscle target. Similar experiments were conducted on neuron B5,a neuron known to be nonselective in synaptogenesis (Haydon and Zoran, 1989). We tested whether transmitter release could be evoked from neuron B5 by nitrophen photolysis following brief periods of assay cell contact (several minutes). Somata of neuron B5 autonomously gain secretory capabilities in the absence of target cell interactions following 1-2 days in cell culture (Haydon, 1988; Haydon et al., 1990). Neuron B5 somata were pressure-injected with 30% calcium-loaded DM-nitrophen and assayed for transmitter release following photolysis. As shown in Figure 5D, photolytic elevation of intracellular calcium caused an increase in the rate of transmitter release from neuron B5 even though it had been cultured in the absence of target cell contacts for 48 hr. Mean release rates for neuron B5 were 0.53 $\pm$0.50 SCs per s before photolysis and 1.60 $\pm$0.36 SCs per s after photolysis.

Discussion

Motoneuron B19 of Helisoma gains the capability of coupling action potentials with the exocytotic release of ACh only after it has contacted appropriate Postsynaptic muscle fibers in cell culture (Figure 2; Zoran et al., 1990). Excitation-secretion coupling is never present in neuron B19 when cultured alone or in contact with novel neuronal targets (Zoran

et al., 1990). Thus, neuron B19 gains functional presynaptic properties in a target-dependent manner.

Experimental elevation of intracellular calcium levels by UV irradiation of a photolabile calcium cage, calcium-loaded DM-nitrophen, allowed an examination of the calcium responsiveness of the secretory apparatus of neuron B19. Photolysis of DM-nitrophen elicited significant increases in transmitter released from B19 somata following sustained periods of appropriate target contact with SLT muscle fibers (Figure 4; Figure 5). Background release rates were not affected by photolytic increases in cytoplasmic calcium when neuron B19 had contacted novel neuronal targets. Therefore, it is unlikely that spontaneous transmitter release is calcium dependent prior to muscle contact. However, cell-cell interactions between neuron B19 and its normal target change this motoneuron's secretory apparatus into a calcium-responsive state.

The contact-dependent regulation of the secretory machinery exhibited by neuron B19 parallels the contact-dependent manner in which this neuron forms only specific synaptic connections in cell culture (Haydon and Zoran, 1989; Zoran et al., 1990). When neuron B19 initially extends neuritic processes it is incapable of forming functional synaptic interactions even though the secretory machinery (as indicated by the presence of spontaneous transmitter release) is present. Action potential-evoked elevations of internal free calcium are effective at triggering synchronous transmitter release only after target recognition. Delayed expression of excitation-secretion coupling has also been demonstrated in the developing rat nervous system, in which calcium-dependent transmitter release is not detected in isolated growth cones until the second postnatal week (Lockerbie et al., 1985; Taylor and Gordon-Weeks, 1989).

In vitro studies of synaptogenesis of neurons isolated from the nervous systems of Aplysia and leech have demonstrated that specific neurons can form either selective or nonselective sets of synaptic con nections (Camardo et al., 1983; Schacher et al., 1985; Arechiga et al., 1986; Vyklicky and Nicholls, 1988). In contrast to neuron B19, the secretory machinery of neuron B5 of Helisoma, a cholinergic neuron that is nonselective in synapse formation (Haydon and Zoran, 1989; Zoran et al., 1990), intrinsically gains a responsiveness

to internal calcium (Figure 5). Thus, as this cell extends neuritic processes, it is competent to release neurotransmitter and form functional synaptic interactions with cholinoceptive targets without specific recognition cues. Studies of neuron B5 regenerating in vivo have demonstrated that neuron B5 forms transient chemical synapses with several novel targets and then gains its specificity by subsequent elimination of novel connections (Haydon and Kater, 1988). This raises the question as to whether the target-induced changes in neuron B19 promote the ability of the whole neuron to release neurotransmitter globally or only at local sites of muscle-contacted membrane. If the neuron as a whole has become secretory, would it then take on the characteristics of a nonselective presynaptic cell such as neuron B5? Mechanisms of synaptic elimination would then be required following global secretory changes in neuron B19 to avoid subsequent formation of inappropriate synaptic connections. The subcellular mechanism underlying action of SILT muscle on neuron B19 is not known. However, several hours of contact with muscle are required for the acquisition of a functional B19 nerve terminal. In contrast, B5 neurites form functional synaptic interactions immediately upon target contact (Haydon and Zoran, 1989; Zoran et al., 1990). We speculate that appropriate target contact may induce the synthesis of a specific presynaptic protein(s) that is critical for excitation-secretion coupling in neuron B19.

Since spontaneous release of neurotransmitter can be detected from neuron B19 with novel target contacts, yet elevated calcium fails to accelerate release rate, it is likely that the "calcium detector" of the secretory machinery a molecule that is crucial for calcium-dependent exocytosis is not expressed until target recognition. Since the roles of most synaptic proteins are as yet unknown, it is not possible to speculate on their possible significance in this system. As probes for synaptic terminal proteins become available for

Helisoma, it will be of great importance to identify those molecules whose appearance is induced by muscle-specific contact, indicating that they are critical components of the calcium-dependent release process.

Experimental Procedures

Animals

Adult, albino (red) pond snails, Helisoma trivolvis, were used in all experiments. Animals were fed lettuce and trout chow daily and were maintained at ambient laboratory temperature.

Spherical Neuronal Somata

Specific neurons were identified and isolated from buccal ganglia that had been pretreated with 0.2% trypsin (type III; Sigma) for 30 min (Hadley et al., 1985; Haydon et al., 1985; Haydon and Zoran, 1990). The isolated neurons were transferred into culture dishes (Falcon #1008) containing 2 ml of defined medium (DM) and 1 % snail hemolymph. DM used for these cultures contained 50% Lebowitz-15 (special order #82-5154EL, without inorganic salts; GIBCO) to which L-glutamine (0.15 mg per ml of DM), gentamicin (50 I.Ll/ml), and inorganic salts were added to give a final concentration of 40 mM NaCl, 1.7 mM KCI, 1.5 mM $MgCl_2$, 4.1 mM $CaCl_2$, 10 mM HEPES (pH 7.4,130 mOsm). Helisoma hemo lymph, which prevents substrate adhesion and discourages neurite extension, was collected from adult animals according to the methods of Hadley and Kater (1983). Neurons cultured in this manner maintain a spherical geometry. In addition, certain neurons (e.g., neuron B5) cultured under these conditions gain the ability to release neurotransmitter from their somatic membrane (Haydon, 1988).

Muscle Cell Dissociation

SLT muscles, innervated by neuron B19 in vivo (Zoran et al., 1989), were dissected whole from adult animals and transferred into DM. SILT muscle masses were then incubated for 4-8 hr in 2 ml of DM containing 1% hemolymph and 4 mg of collagenase (CLS IV; Worthington Biochemical Corp.). The dissociation flask was gently agitated in a 34 C water

bath. Following collagenase incubation, the tissue was washed with DM and shaken by hand to dissociate the muscle. Single SLT muscle fibers were transferred to nonadhesive culture dishes and stored at room temperature until used. Muscle dissociation procedures and single fiber physiology have been previously described (Zoran et al., 1989, 1990).

Nerve-Muscle Cultures

Single SLT muscle cells (100-1000 pm in length and 5-15 pm in width) were plated in clusters of 5 or 6 fibers on poly-L-lysine coated culture dishes containing 2 ml of DM. Muscle fibers were allowed 1-2 hr to adhere sufficiently to the substrate. Spherical somata were then manipulated into contact with several muscle fibers and allowed to adhere. The nerve-muscle cultures were maintained for 48 hr of contact in 1% hemolymph-conditioned DM. These nerve-muscle cocultures were then electrophysiologically assessed, using ACh-sensitive assay cells (Haydon and Man-Son-Hing, 1988; Haydon and Zoran, 1989), to evaluate the influence of muscle on the neuron's ability to release neurotransmitter.

In control experiments, neuronal somata were cultured under identical conditions to the appropriate nerve-muscle cultures except that presynaptic neuronal somata were cultured alone or in contact with a novel neuronal target (another neuron B19) for the 48 hr period. We use the terms "appropriate" and "novel" targets to identify cells that were and were not connected by chemical synapses in the unperturbed adult nervous system, respectively.

ACh-Sensitive Assay Cells

ACh-sensitive assay cells were used for detection of ACh release from presynaptic neurons. Assay neurons (neuron B19) were cultured for 2 days in 1% Helisoma hemolymph in DM, thereby preventing adhesion and discouraging neurite extension. These spherical cells were sensitive assays of ACh release (Haydon, 1988; Haydon and Man-Son-Hing, 1988; Haydon and Zoran, 1989). Cells were manipulated into contact with presynaptic neuronal somata and allowed to contact for 30 min prior to electrophysiological monitoring of chemical transmission.

Electrophysiology

Cells were viewed on an inverted Nikon diaphot microscope. Glass microelectrodes, filled with 1.5 M KCI (or in some cases with 5% Lucifer yellow), were used for presynaptic neuronal penetrations and had resistances of 10-20 MΩ. The electrodes were connected to the bridge-balance amplifiers of a Dagan 8800 Total Clamp. ACh-sensitive assay cells were voltage-clamped in the whole-cell mode with patch pipettes filled with an internal solution consisting of 50 mM KCI, 5 mM MgCl,, 5 mM EGTA, and 5 mM HEPES (pH 7.3). Patch pipettes used in brief contact experiments had direct current resistances of 2-4 MΩ. Records were collected with an Axopatch-1C (Axon instruments, California) patch-clamp amplifier and visualized on a Tektronix 5111A storage oscilloscope. The data were stored on videotape using a Unitrade (Toshiba) digital data acquisition system.

Release of neurotransmitter was classified as SSCs when they were not correlated with action potentials in the presynaptic cell and as ESCs when elicited by action potentials in the presynaptic cell. SCs were considered evoked only if they followed presynaptic action potentials at a constant latency within a given preparation. SC waveforms and criteria for SC identification have been previously discussed by Zoran et al. (1990).

Calcium Current Analysis

Spherical neuronal somata were cultured as described above. Following 48 hr of culture alone, with novel neuronal contact or appropriate muscle contact, neurons were voltage-clamped using an Axopatch-1C patch-clamp amplifier for calcium current examination. The patch-pipette solution contained 35 mM CsCl, 5 MM $MgCl_2$, 5 mM EGTA, and 5 mM HEPES. The neurons were bathed in 4.1 mM $CaCl_2$, 1-5 MM $MgCl_2$, 1.7 mM KCl, 10 mM 4-aminopyridine, 30 mM tetraethylammonium bromide, 30 mM sucrose, 10 mM HEPES (pH 7.3). Linear leakage and capacity currents were digitally subtracted from depolarizing current traces using pCIamp software (Axon Instruments). Calcium current clensities were calculated using cell surface areas estimated from measurements of the whole-cell membrane capacitance.

Caged Calcium Photolysis

Neuron B19 used in photolysis experiments was plated on to poly-L-lysine-coated glass-bottom culture dishes. Culture conditions and electrophysiological methods were identical to those described above. Caged-calcium solutions containing 75 mM DM-nitrophen (Calbiochem) in distilled water were pressureinjected into presynaptic neuron B19 through glass microelectrodes. The tips of the pressure pipettes were broken by gentle manipulation against a glass coverslip. Pressure pulses of 5-20 ms (30 psi) were applied using an IM-200 (Narishige, USA) pressure injection system. Injection solutions of 75 mM DM-nitrophen contained either calcium-free or 30% calcium-loaded DM-nitrophen (21.6 mM $CaCl^2$

Neurons were viewed with a Nikon diaphot microscope using a 40x oil immersion objective. Photolysis of DM-nitrophen was accomplished using UV light delivered from a 100 W mercury vapor lamp focused onto the cell by the epifluorescence 40x objective. Exposure durations of 3 s were controlled by manually opening a shutter. In most experiments, the intensity of illumination was attenuated using a 17% transmittance neutral density filter. The average rate of release (SCs per s) was calculated for a 10 s period immediately before and after photolysis.

Estimation of Intracellular Calcium Levels

Estimates of intracellular free calcium were made during these experiments to confirm that photolysis of DM-nitrophen brought about an increase in presynaptic calcium levels. The calcium sensitive fluorescent dye fluo-3 (1 mM; Minta et al., 1989; Kao et al., 1989) was included with DM-nitrophen in the injection solution. Successful injection of the DM-nitrophen solution was readily judged by an increase in the fluorescence of the cell with increases in the internal level of fluo-3. Fluorescence of fluo-3 (excitation, 480 nm; emission, 515 nm; dichroic, 510 nm) was recorded with a photomultiplier using the UMANS system (Dr. Chester M. Regen, Urbana, IL). Estimates of intracellular calcium were made according to the procedures of Kao et al. (1989) using calcium ionophore and $MnCl_2$ Estimates were made for pre-photolysis and post-photolysis periods on all cells tested.

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References

Arechiga, H., Chiquet, M., Kuffler, D. P., and Nicholls, J. G. (1986). Formation of specific connections in culture by identified leech neurones containing serotonin, acetylcholine, and pepticle transmitters. J. Exp. Biol. 126, 15-31.

Camardo, J, E., Proshansky, F., and Schacher, S. (1983). Identified Aplysia neurons form specific chemical synapses in culture. Neurosci. 3, 2614-2620.

Chow, I., and Poo, M.-m. (1985). Release of acetylcholine from embryonic neurons upon contact with muscle cell. J. Neurosci. 5, 1076-1082.

Cohan, C. S., Connor, J. A., and Kater, S. B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J. Neurosci. 5, 1076-1082.

Delaney, K. R., and Zucker, R. S. (1990). Calcium released by photolysis of DM-nitrophen stimulates transmitter release at squid giant synapse. J. Physiol. 426, 473-498.

Easter, S. S., Purves, D., Rakic, P., and Spitzer, N. C. (1985). The changing view of neural specificity. Science 230, 507-511.

Evers, J., Laser, M., Sun, Y.-a., Xie, Z.-p., and Poo, M.-m. (1989). Studies of nerve-muscle interactions in Xenopus cell culture: analysis of early synaptic currents. J. Neurosci. 9, 1523-1539.

Hadley, R. D., and Kater, S. B. (1983). Competence to form electrical connections is restricted to growing neurites in the snail, Helisoma. J. Neurosci. 3, 924-932.

Hadley, R. D., Bodnar, D. A., and Kater, S. B. (1985). Formation of electrical synapses between isolated, cell cultured Helisoma neurons requires mutual neurite elongation. J. Neurosci. 5, 3145-3153.

Haydon, P. G. (1988). Chemical synapses form between isolated neuronal somata in the absence of neurite extension. J. Neurosci. 8, 1032-1038.

Haydon, P. G., and Kater, S. B. (1988). The differential regulation of formation of electrical and chemical connections in Helisoma. J. Neurobiol. 19, 636-655.

Haydon, P. G., and Man-Son-Hing, H. J. (1988). Low- and highvoltage-activated calcium currents: their relationship to the site of neurotransmitter release in an identified neuron of Helisoma. Neuron 1, 919-927.

Haydon, P. G., and Zoran, M. J. (1989). Formation and modulation of chemical connections: evoked acetylcholine release from growth cones and neurites of specific identified neurons. Neuron 2, 1483-1490.

Haydon, P. G., and Zoran, M. J. (1990). Chemical synapses in culture. In Cellular and Molecular Neurobiology: A Practical Approach. H. Wheal and J. Chad, eds. (Oxford: IRL Press), in press.

Haydon, P. G., Cohan, C. S., McCobb, D. P., Miller, H. R., and Kater, S. B. (1985). Neuron specific growth cone properties as seen in identified neurons of Helisoma. J. Neurosci. Res. 13, 285-300.

Haydon, P. G., Zoran, M. J., Man-Son-Hing, H. J., Sievers, E. M., and Doyle, R. T. (1990). A relation between specificity and the acquisition of presynaptic properties. J. Physiol. (Paris) 84, 111-120.

Kao, J. P. Y., Harootunian, A. T., and Tsien, R. Y. (1989). Photochemically generated cytosolic calcium pulses and their detection by fluo-3. J. Biol. Chem. 264, 8179-8184.

Kaplan, J. H., and Ellis-Davies, G. C. R. (1988). Photolabile chelators for rapid photolytic release of divalent cations. Proc. Natl, Acad. Sci. USA 85, 6571-6575.

Lockerbie, R. 0., Gordon-Weeks, P. R., and Pearce, B. R. (1985). Growth cones isolated from developing rat forebrain: uptake and release of GABA and noradrenaline. Dev. Brain Res. 21, 265-275.

Minta, A., Kao, J. P. Y., and Tsien, R. Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. 264, 8171-8178.

Schacher, S., Rayport, S. G., and Ambron, R. T. (1985). Giant Aplysia neuron R2 reliably forms strong chemical connections in vitro.]. Neurosci. 5, 2851-2856.

Sperry, R. W. (1963). Chemoaffinityin the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA 50,703-709.

Sun, Y.-a., and Poo, M.-m. (1987). Evoked release of acetylcholine from the growing embryonic neuron. Proc. Natl. Acad. Sci. USA 84, 2540-2544.

Takahashi, T., Nakajima, Y., Hirosawa, K., and Onodera, K. (1987). Structure and physiology of developing neuromuscular synapses in culture. J. Neurosci. 7, 473-481.

Taylor, J., and Gordon-Weeks, P. R. (1989). Developmental changes in the calcium dependency of gamma-aminobutryic acid release from isolated growth cones: correlation with growth cone morphology. J. Neurochem. 53, 834-843.

Vyklicky, L,, and Nicholls, J. G. (1988). Specificity of connections formed by nociceptive cells of the leech in tissue culture. J. Exp.Biol. 134, 17-26.

Zoran, M. J., Haydon, P. G., and Matthews, P. J. (1989). Aminergic and peptidergic modulation of an identified motor pathway in the gastropod mollusc, Helisoma. J. Exp. Biol. 142, 225-243.

Zoran, M. J., Doyle, R. T., and Haydon, P. G. (1990), Target-dependent induction of secretory capabilities in an identified motoneuron during synaptogenesis. Dev. Biol. 138, 202-213.

APPENDIX C. CONTACT-DEPENDENT REGULATION OF N-TYPE CALCIUM CHANNEL SUBUNITS DURING SYNAPTOGENESIS

Contact- Dependent Regulation of N-Type Calcium Channel Subunits during Synaptogenesis

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ABSTRACT

The developmental regulation of the N-type calcium channel during synaptogenesis was studied using cultured rat hippocampal neurons to elucidate the roles of extrinsic versus intrinsic cues in the expression and distribution of this channel. Prior to synapse formation, a1B and 83 subunits of the N-type calcium channel were distributed diffusely throughout neurites, growth cones, and somata. As synaptogenesis proceeded, the subunit distributions became punctate and colocalized with the synaptic vesicle protein synaptotagmin. Isolated neurons were also examined to test for the requirement of extrinsic cues that control N-type calcium channel expression and distribution. These neurons expressed N-type calcium channel subunits, but their distributions remained diffuse. Functional ω-conotoxin GVIA-sensitive channels were expressed in isolated neurons, although the distribution of $\alpha_{1\beta}$ subunits was diffuse. The distribution of the ant subunit and synaptotagmin only became punctate when neuron-neuron contact was allowed. Thus, the expression of functional Genotype calcium channels is the result of an intrinsic program while extrinsic regulatory cues mediated by neuronneuron contact are required to control their distribution during synaptogenesis.

INTRODUCTION

Influx of calcium through voltage-dependent calcium channels plays an integral role in vesicle fusion during synaptic transmission. In neurons, many calcium channel subtypes have been identified (Tsien et al., 1988; Bean, 1989; Llinas et al., 1992; Miller, 1992). Thus far, N-, P-, and Q-type calcium channels have been implicated in evoking fast neurotransmitter release (Wheeler et al., 1994; Luebke et al., 1993; Takahashi and Momiyama, 1993; Turner et al., 1993). Pharmacologically, the N-type channel can be distinguished from other channel types by its sensitivity to ω-conotoxin GVIA (ωCTX GVIA), a peptide toxin isolated from the snail *Conus geographus* (Olivera et al., 1984).

ω-CTX GVIA binds irreversibly to the N-type calcium channel, blocking calcium influx and transmitter release in several vertebrate neurons (Boland et al., 1994).

Witcher et al. (1993b) used w-CTX GVIA to purify the N-type calcium channel. They found that it contains four distinct subunits of molecular weight 230, 140, 95, and 57 kD. Antibodies which recognize specific regions of these subunits demonstrated that the α_{1b} and β_3 subunits of the N-type calcium channel are immunologically distinct from those of dihydropyridine- sensitive calcium channels. Consistent with a role in transmitter release, the N-type calcium channel has been localized to the release face of nerve terminals using fluorescently labeled w-CTX GVIA (Robitaille et al., 1990; Cohen et al., 1991; Torri Tarelli et al., 1991). In addition, the spatial distribution of calcium channels at the single channel level has been studied. We have observed, using atomic force microscopy, that ω–CTX GVIA binding sites are organized in clusters and short linear or parallel arrays on the release face of presynaptic nerve terminals isolated from chick ciliary ganglia (Haydon et al., 1994).

The mechanism of regulation of calcium channel distribution during synapse formation is undefined. However, it is clear that the N-type channel is critical for exocytosis immediately after synaptogenesis, although with further synaptic maturation other channel types become more important in controlling exocytosis (Scholz and Miller, 1995; Verderio et al., 1995). In the present study, we asked whether neuron-neuron contact regulates the distribution of N-type calcium channels in the presynaptic terminal of cultured embryonic rat hippocampal neurons during synaptogenesis. These experiments demonstrate many parallels between the regulation of acetylcholine receptors at the neuromuscular junction and calcium channels in the presynaptic nerve terminal. For example, we find that prior to target interaction, the subunits of the N-type calcium channels are synthesized and functional ω-CTX GVIA- sensitive calcium channels are assembled. Furthermore, the distribution of these channels remains diffuse until cues provided by synaptic contact cause them to reorganize into discrete punctate clusters

MATERIALS AND METHODS

Hippocampal Cultures

Hippocampi dissected from E17 or E18 rat fetuses were dissociated by trypsinization [0.025% trypsin in Ca^{+2}/ Mg^{+2} Earle's balanced salt solution (EBSS)] for 15 min followed by trituration in modified minimal essential medium (MEM) (Eagle's minimum essential medium, 2 mM glutamine, I mM sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin) supplemented with 10% fetal bovine serum. The cells were then incubated for 30 min at 4°C before plating onto 12-mm-diameter circular glass coverslips coated with covalently bound poly-L-lysine (Scholz and Miller, 1995). Coverslips were then placed into 35-mm Falcon culture dishes containing modified MEM supplemented with N2 (3 coverslips/ dish). After 24 h, the medium was replaced with MEM supplemented with N2 and the cultures were maintained in a humidified 5% C02/95% room air incubator at 37C. This culture method provided highly enriched pyramidal neuron cultures with <3% astrocytes, as verified by immunocytochernistry for glial fibrillary-associated protein (results not shown). Cells were plated at a density of 60-80 cells/mm2 such that by approximately 48 h, the majority of neurons had made contact with other neurons. For experiments where isolated neurons were necessary, low-density cultures were prepared (5-10 neurons/ mm^2). With this method, it was possible to clearly identify isolated neurons. To enhance long-term survival and to control for possible diffusible factors that may affect synaptic development, each coverslip with low-density neurons was cultured with two normal density coverslips in the same 35-mm culture dish.

Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

Expression of the N-type calcium channel alpha subunit gene was determined by both single-cell RT-PCR and amplification of cDNA obtained by reverse transcription of mRNA from rat hippocampal neurons in primary culture or acutely isolated hippocampal slices. The mRNA was purified using oligo-dT beads (Oligotex; Qiagen). The poly-A transcript was reverse-tran scribed to firststrand cDNA using a First Strand cDNA Synthesis Kit (Pharmacia) and 10 pmol of a specific antisense primer of α_{1b} (TGC TGA GTC CCA AAG TGC).

Aliquots of cDNA template were added to the PCR reaction mixture with 10 pmol each of α_{1b} sense (TAG CCA GGT GTC CCA TCA) and antisense primers, 100 mM dNTPs,

2 MM MgC12, 0.5 U *Taq* polymerase. After 30 cycles, 5 μL of PCR product was used as the template for another PCR reaction using 10 pmol each of specific primers internal to the previous primers: sense (ACC ACC ACC GCT GCC ACC), antisense: (TAG CCA TTG GGT ACA CGG).

Single-Cell PCR

Single hippocampal neurons were drawn into a glass patch-clamp pipette using negative pressure. The pipette contents were expelled into a test tube and mixed with 3.5 μL of first-strand cDNA synthesis mixture containing 45 mMTris (pH 8.3), 68 mM KCI, 15 mM dithiothreitol (DTT), 9 mM MgCl, 0.08 mg/mL bovine serum albumin (BSA), 1.8 mM of each dNTP, 10 pmol of specific α_{1b} antisense primer (see above), and 10 U of murine reverse transcriptase. The mixture was incubated at 37'C for I h. The reaction was terminated by incubating at 95°C for 2 min. The cDNA from the first-strand synthesis reaction was subjected to PCR as described above. Two types of controls were utilized. First, reactions were performed in the absence of reverse transcriptase. Second, we performed the complete RT-PCR reaction on saline that was aspirated into a patch pipette from a region adjacent to a neuron. In all experiments, these controls were negative. All results were generally replicated in at least three independent experiments.

Production and Purification of Specific Polyclonal Antibodies to the α_{1b} and β_3 Subunits

Polyclonal antibodies against the N-type Ca^{2+} channel complex and specific α_{1b} and β_3 GST fusion proteins were produced as previously described (Witcher et al., 1993a). Specific sheep polyclonal antibodies (sheep 46) were produced against the purified N–type Ca^{2+} channel complex, and sheep polyclonal antibodies (sheep 49) were generated against an unique 63 C-terminal GST fusion protein. Specific rabbit polyclonal antibodies (rabbit 95) were also produced against an a1B GST fusion protein (Witcher et al., 1993a). Both the α_{1b} and β_3, GST fusion proteins were covalently coupled to CNBr-Sepharose as previously described (Witcher et al., 1995). These resins were used to affinity-purify specific polyclonal antibodies to α_{1b} and β_3 subunits. Briefly, serum containing specific polyclonal antibodies to the α_{1b} and β_3 subunits was diluted 1:10 with 20 mM MOPS, 150 mM NaCl, pH 7.4, and

placed over the α_{1b} or β_3 GST fusion protein Sepharose columns. The Sepharose columns were then extensively washed with 20 mM MOPS, 150 mM NaCl, pH 7.4, and the polyclonal antibodies were eluted from the columns with 20 mM glycine, 150 mM NaCl, pH 2.4. The eluted fractions were immediately neutralized with 1M MOPS, pH 7.6. Affinity-purified sheep 49 and rabbit 96 polyclonal antibodies were also placed on a GST Sepharose column to remove any GST antibodies. Finally, the affinity-purified polyclonal antibodies were concentrated and dialyzed in phosphate-buffered saline (PBS) containing 20% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.001% sodium azide.

Immunohistochemistry

Cultures were fixed in Zamboni's fixative for 60 min at room temperature followed by multiple rinses in 0.1 M PBS. After additional washing with 0.05 M Tris-buffered saline, the coverslips were incubated in 0.02% Triton-X 100 (5 min, 27 ^{0}C) and blocked in 1% BSA and 1.5% normal rabbit or goat serum (60 min, 37 ^{0}C). Following overnight incubation (4 ^{0}C) with anti-α_{1b} or β_3 (1:500), visualization was accomplished using a biotinylated goat anti-rabbit immunoglobulin G (IgG) or rabbit anti-sheep IgG (60 min, 27 ^{0}C) and avidin conjugated with fluorescein or rhodamine. The coverslips were then mounted onto microscope slides using glycerol containing n-propyl gallate. For double-label experiments, coverslips were incubated with either anti-MAP2 (AP-20, 1:1000), or anti-synaptotagmin (CL41.1, 1:500) in addition to incubation with the anti-calcium channel subunit antibodies. For these experiments, MAP2 and synaptotagmin were visualized using a rhodamine-conjugated goat anti-mouse IgG. Anti-synaptotagmin (CL41.1) was generously provided by Dr. R. Jahn, MAP2 (clone AP-20) was from Boehringer, and anti-GFAP from Sigma (St. Louis, MO). Control experiments in which primary antibodies were omitted were negative.

Calcium Imaging

The presence of voltage- sensitive calcium influx in neurons >4 days in culture was examined using Fura-2 and ratiometric imaging techniques as previously described

(Grynkiewicz et al., 1985; Basarsky et al., 1994). After loading with Fura-2/AM (2 μM for 45 min at 37^0C and de-esterifying for an additional 45 min, ratio images (350 nm/380 nm) were acquired at 5-s intervals using a CH250 Photometrics liquid-cooled CCD camera and Metafluor software (Universal Imaging). Two images were acquired for baseline Ca^{2+} levels, then two additional images were taken while neurons were depolarized with 50 mM K+ saline. One final image was taken I min after normal saline flow was restored. A second depolarization was performed after a 10-min recovery period in flowing normal saline. The second depolarization involved either control (normal saline) or pharmacological solutions. The results of the two units of depolarization were compared to assess the effect of the control versus test solutions on Ca^{2+} accumulation.

Measurement of Calcium Currents

Calcium currents were measured by voltage-clamping cultured hippocampal neurons in the whole-cell mode. Patch pipettes were pulled in two stages on a Narishige PP-83 puller from 1.5-mm O.D.-I.12-mm I.D. omega dot borosilicate capillaries (FHC, Brunswick, ME). Their resistance was between 5 and 8 MΩ when filled with internal recording solution. The shank of the electrodes was coated with wax to reduce capacitance. No fire polishing was used. The internal solution consisted of (in mM): tetraethylammonium chloride (TEA-Cl) 117, MgC12 4.5, ethylene glycol-bis(b-aminoethylether) *NNN',N'-tetraacetic acid* (EGTA) 9, Hepes 9, phosphocreatine, adenosine 5'-triphosphate magnesium salt (Mg-ATP) 4, phosphocreatine 14, guanosine 5'-triphosphate tris salt (Tris-GTP) 0.3 [pH 7.4 adjusted with tetraethylammonium hydroxide (TEA-OH)]. The external recording medium was (in mM): BaC12 10 or 25, TEA-Cl 160 or 145, Hepes 10 (pH 7.4 adjusted with TEA-OH). The data were acquired and analyzed with pCIamp software (Axon Instruments).

Drugs.

Drugs used were TEA-Cl, TEA-OH, barium chloride, EGTA, phosphocreatine, Mg-ATP, and TrisGTP, obtained from Sigma. ω-CTX GVIA was obtained from Peptides International (Louisville, KY).

RESULTS

mRNA for the α_{1b} Calcium Channel Subunit Is Present in Immature Neurons

Functional synaptic transmission can be detected in 17 to 18-day embryonic hippocampal pyramidal neurons after 7-8 days in culture (Doyle et al., 1996; Scholz and Miller, 1995). Around the time of synapse formation, N-type calcium channels have been shown to be the primary class of channel linked to transmitter release (Scholz and Miller, 1995). We focused on the developmental aspects of expression and function of the N-type channel and considered neurons earlier than 6 days in culture to be synaptically immature. We initially asked when mRNA for the α_{1b} subunit was present utilizing RT-PCR. We performed RT-PCR on mass-dissociated neurons from day E18 rats at times varying from zero to 14 days in culture [Fig. 1(A)]. The presence of mRNA for the α_{1b} subunit was detected at all ages examined. To confirm that the α_{1b} transcripts originated from pyramidal neurons, we performed single-neuron RT-PCR on isolated single pyramidal neurons after varying times in culture [Fig.1(A)]. Again, α_{1b} mRNA could be detected at all ages examined. Thus, at early times points in culture, mRNA encoding the α_{1b} subunit of the N-type calcium channel is present in hippocampal pyramidal neurons.

Functional N-Type Calcium Channels Are Present in Synaptically Immature Neurons

The presence of the α_{1b} mRNA does not necessarily mean that functional N channels have been assembled and inserted into the neuronal membrane. To test for the presence of functional N channels, we measured macroscopic calcium currents using patch-clamp recordings in the whole-cell mode. To rapidly generate current-voltage relationships, we used a slow ramp from - 100 to + 50 mV as a command. Ramps were obtained before and after 5

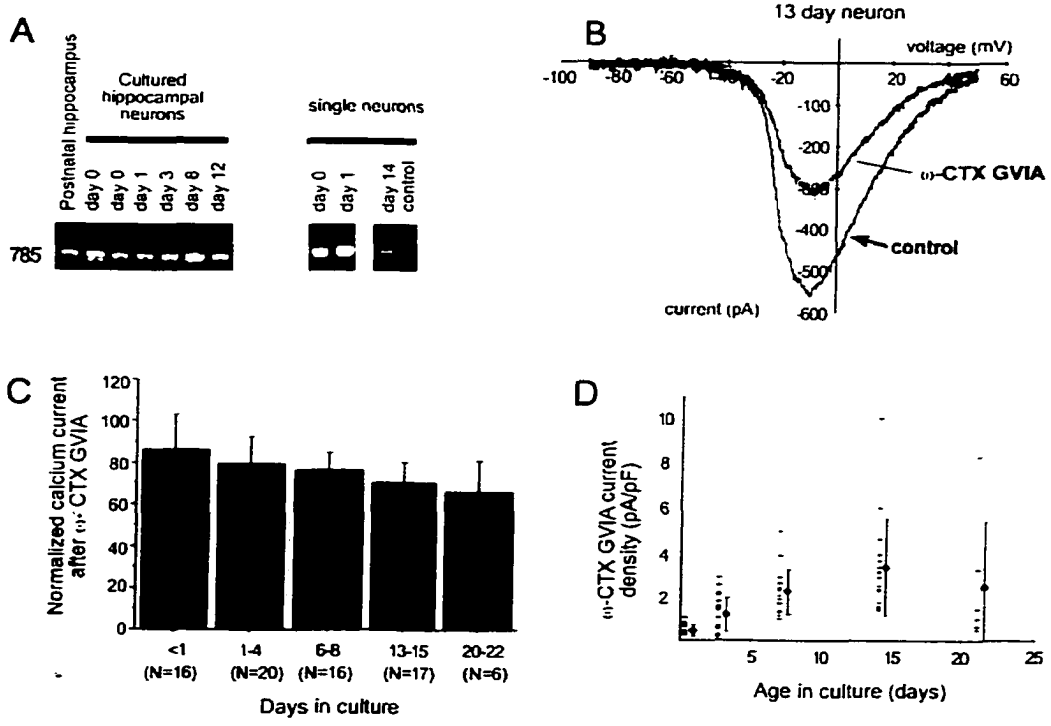

Figure 1. Functional N-type calcium channels are synthesized and inserted into the neuronal membrane prior to synapse formation. (A) m-RNA for the α1B calcium channel subunit was detected using RT-PCR from postnatal hippocampus, embryonic hippocampal neuronal cultures (at least six independent experiments at each time point, with positive results in 67 - 100% of reactions) and from single cultured embryonic hippocampal pyramidal neurons. Control was a single 14-day neuron processed without reverse transcription. While it appears that less product is generated after 14 days in culture in single-cell PCR, quantitative comparisons are not appropriate under our conditions. (B) Whole-cell calcium currents recorded from a neuron after 13 days in culture before and after exposure to 5 mM ω-CTX GVIA. (C) Summary of the actions of ω-CTX GVIA on whole-cell calcium currents in neurons at different times in culture. Responses were normalized to the response to pressure ejection of external barium saline onto the soma of neurons of the same age. The response to ω-CTX GVIA was significantly different from that to barium saline at all ages examined ($p < 0.015$ for neurons <1 day in culture; $p < 0.001$ for all other ages; two-way ANOVA with Scheffe' post hoc comparison). (D) The magnitude of the ω-CTX GVIA- sensitive calcium current density (i.e., N-type calcium current) increases with increasing age in culture. (Individual data points are indicated by dashes; offset to the right is the mean and standard deviation for each age.)

mM ω-CTX GVIA was applied to the neuronal soma by pressure ejection [Fig.1(B)]. Administration of ω-CTX GVIA produced a decrease in the whole-cell calcium current at all ages of neurons examined [23 ± 14%; mean ± standard deviation (S.D.), n = 75]. An effect of ω-CTX GVIA could be demonstrated as early as 2-3 h after neurons were isolated and placed into culture. The actions of ω-CTX GVIA were significantly different from the effects of pressure ejection of external barium saline onto the soma at all ages examined [$p < 0.0151$

for neurons <1 day; $p < 0.000\ 1$ for all other ages; two-way analysis of variance (ANOVA) with Scheffe' post hoc comparison] [Fig.1(C)]. While a1B subunit mRNA and ω-CTX GVIA-sensitive calcium currents are present from the time of plating in culture, the magnitude of the ω-CTX GVIA-sensitive current density (ω-CTX GVIA current density) increases with age in culture [Fig.1(D)]. However, the relative contribution of the ω-CTX GVIA-sensitive calcium current to the whole-cell calcium current was not affected by the age of the neuron ($p = 0.6156$; two-way ANOVA with Scheffe' post hoc comparison).

Functional N-Type Calcium Channels Are Present in Neurites

While patch-clamp experiments demonstrated the presence of functional N-type calcium channels in the soma of hippocampal neurons, they did not allow us to determine whether functional channels were present in neurites. We used calcium imaging methods to determine the ω-CTX GVIA-sensitive contribution to depolarization-induced calcium accumulation in neurites. Bath application of ω-CTX GVIA (1mM) caused a 51 ± 4% decrease in calcium accumulation ($n = 20$) (Fig. 2) measured in neurites of neurons maintained in culture for 4 days. In comparison to voltage-clamp experiments, ω-CTX GVIA produced a more substantial reduction in calcium accumulation than expected. Perhaps this results from a differential contribution of distinct calcium channel subtypes in neurites compared to somata. Alternatively, this quantitative difference may be due to the sustained period of depolarization (~10 s) employed in the calcium imaging experiments. Nonetheless, these data suggest that at this early time point in culture, prior to synaptogenesis, the mRNA detected by RT-PCR gives rise to functional ω-CTX GVIA-sensitive N-type calcium channels in the neurites of synaptically immature neurons.

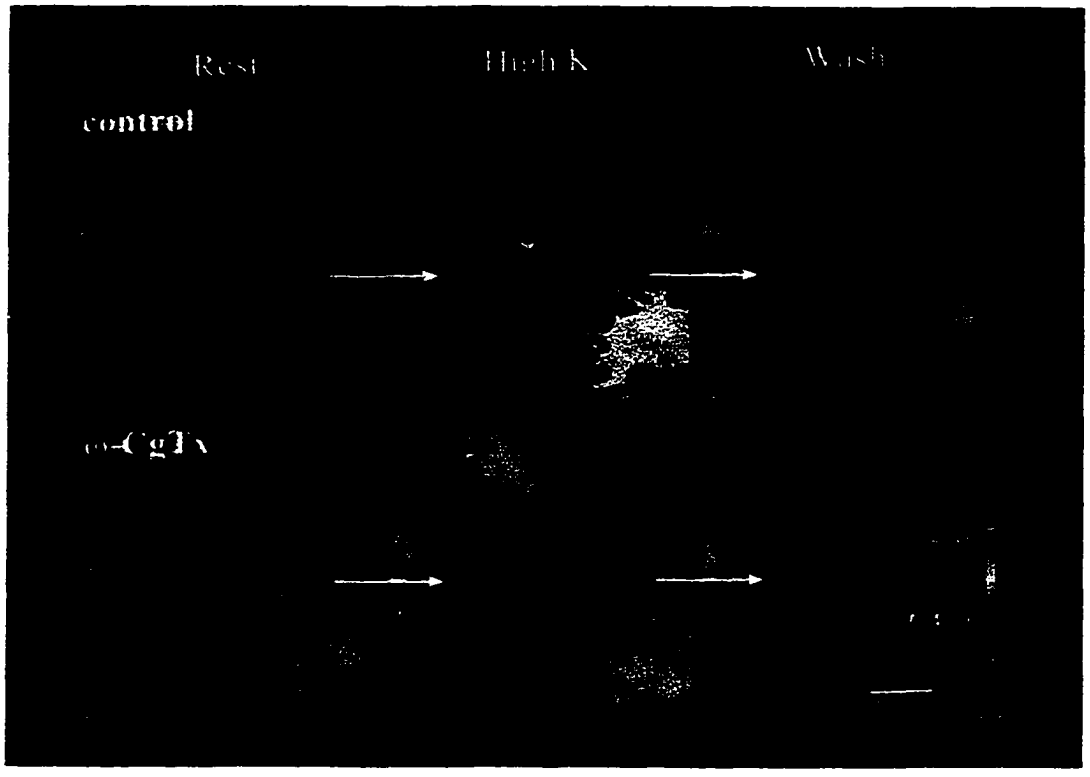

Figure 2. ω-CgTx GVIA reduces depolarization-dependent calcium accumulation in somata and neurites of hippocampal neurons. (Top) Sequential ratiometric images displaying estimated calcium levels of hippocampal neurons. Depolarization of neurons with high-K^+ saline elevates calcium throughout the neurons. (Bottom) Addition of 1 μM ω-CgTx reduces the extent of depolarization-dependent calcium accumulation both within the neurites and somata.

Immunocytochemical Characterization of N-Type Calcium Channel Subunits in Developing Neurons

To further evaluate the distribution of N-type channels prior to synapse formation, we used immunocytochemistry to examine the expression and distribution patterns of the α_{1b} and β_3 subunits of this calcium channel. In young, synaptically immature neurons (=4 days in culture), the immunoreactivity for both subunits was largely diffuse throughout the soma and neurites Fig. 3 (A,C)]. There were, however, notable regions of enhanced neurite staining. These regions were frequently observed at sites of soma or neurite contact. Synaptically mature neurons 12 days in culture also had diffuse staining throughout the soma and apical dendrite, but staining in neurites was largely punctate [Fig. 3(b,d)].

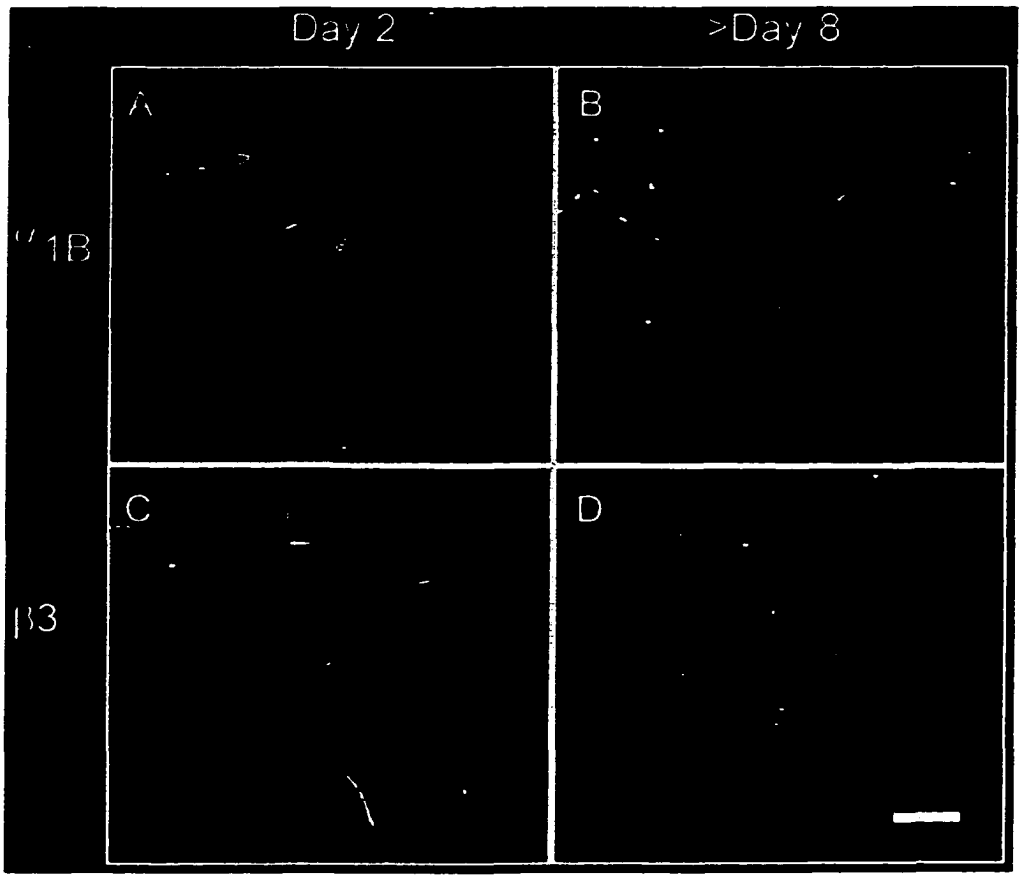

Figure 3. Expression and distribution of the α_{1b} and β_3 subunits of the N-type calcium channel during development. (A) At 2 days in culture, α_{1b} is expressed, but is distributed diffusely throughout the neuron. (B) By 8 days in culture, α_{1b} immunoreactivity becomes punctate in neurites. (C,D) Distributions of the β subunit parallel the distribution of the α_{1b} subunit. Bar = 20 μM.

To determine whether the α_{1b} subunit is localized in the axons or dendrites of mature neurons, we used immunocytochemistry to double label for the dendritic marker MAP2 and the α_{1b} subunit. While the α_{1b} subunit was colocalized in neurites with MAP2, there was also α_{1b} subunit immunoreactivity in many neurites that were MAP2 negative (data not shown). This nondendritic α_{1b} subunit immunoreactivity was generally punctate in appearance. Double labeling using anti-α_{1b} and anti - synaptotagmin revealed that synaptotagmin and the N-type calcium channel subunit α_{1b} are colocalized in mature neurons at least to the extent resolved by the diffraction- limited optics of fluorescence microscopy. Staining profiles in

the neurites of neurons after 8 days in culture were punctate for both α_{1b} and synaptotagmin (Fig. 4).

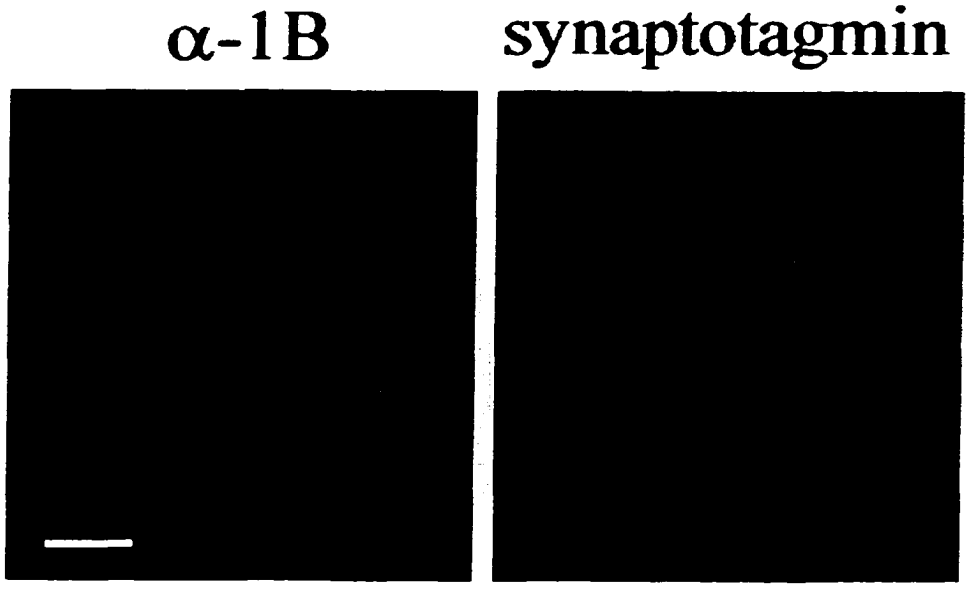

Figure 4. Double-label immunocytochemistry for α_{1b} and synaptotagmin. After 8 days in culture, α_{1b} and synaptotagmin colocalize. Bar = 20 μm.

A Punctate, Synaptic Distribution of the α_{1b} Subunit Requires Neuron-Neuron Interactions

Neuron-neuron contact is likely to play a key role in the regulation of N-type calcium channel expression and distribution (Jones et al.,1989). To test this hypothesis, we cultured hippocampal neurons under conditions which resulted in either isolated neurons or neurons with neuron-neuron contacts.

Immunocytochemistry was then performed to localize the a, subunit of the N-type calcium channel. After 8 days in culture, isolated neurons had α_{1b} staining that was diffuse and nonpunctate [Fig. 5(a)]. Whole-cell recording from such isolated neurons revealed the presence of functional calcium currents sensitive to ω-CTX GVIA (32.0 ± 7.6%; n = 6). In contrast to the diffuse immunostaining in isolated neurons, punctate α_{1b} staining was seen in the neurites of neurons 8 days in culture that were allowed to form neuron-neuron contacts [Fig. 5(d)].

Immunocytochemistry to localize synaptotagmin was also performed on isolated hippocampal neurons. Like the α_{1b} subunit, punctate synaptotagmin staining was detected in cultures where neurons were allowed neuron-neuron contact [Fig. 5(e)], but was diffuse and immature in isolated neurons [Fig. 5(b)]. These data suggest that isolated neurons are capable of expressing the α_{1b} subunit of the N-type calcium channel and synaptotagmin, but extrinsic signals are required to control their distribution.

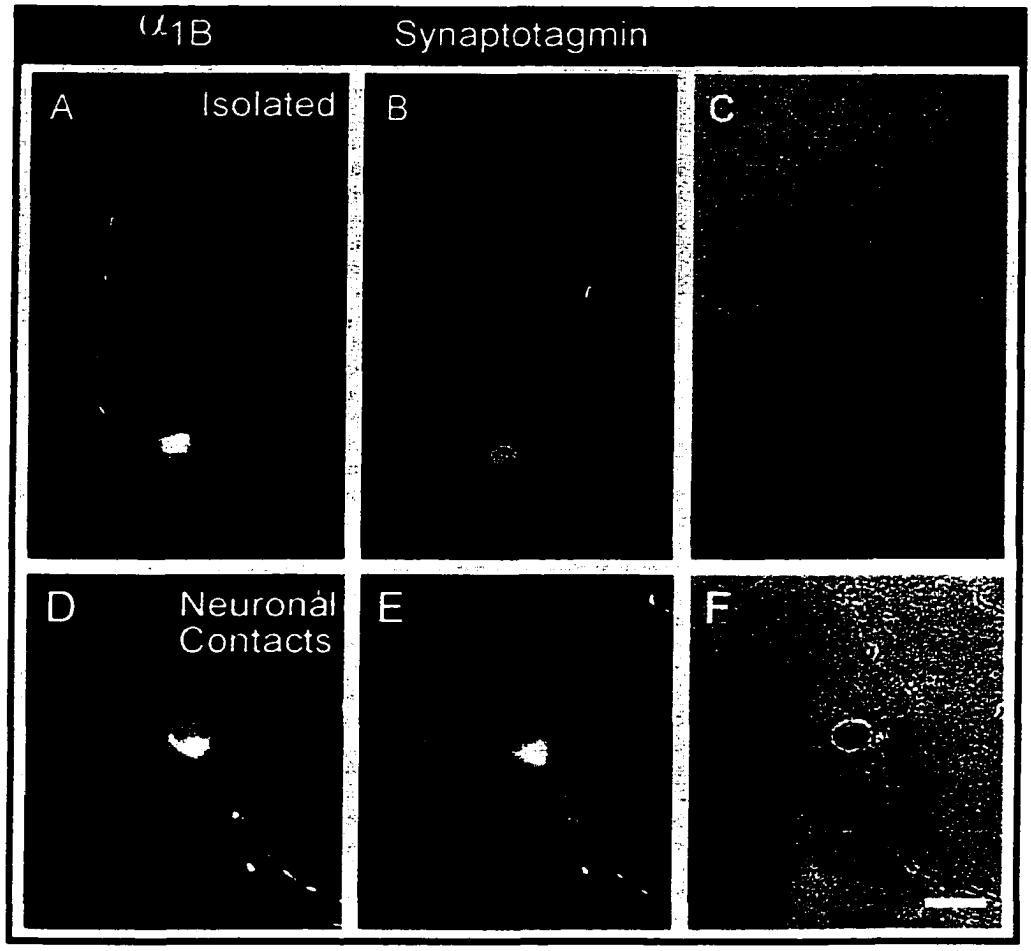

Figure 5. α_{1b} and synaptotagmin expression and distribution in isolated neurons and in neurons that were allowed neuron-neuron contacts. (A, B) α_{1b} and synaptotagmin immunoreactivities were distributed diffusely in isolated hippocampal neurons that were grown for 8 days in culture. (D,E) In contrast, α_{1b} and synaptotagmin immunoreactivities were punctate in the neurites of neurons that were grown in parallel cultures where neuron-neuron contacts were allowed. Bar = 20 μm.

DISCUSSION

Our understanding of the developmental mechanisms that regulate the assembly of a functional synaptic connection has, in large part, been due to studies performed at the experimentally accessible neuromuscular junction. This system has yielded much information regarding the control of the postsynaptic acetylcholine receptor which we now know is regulated by multiple signals. For example, agrin, which can be derived from innervating neurons, induces the accumulation of the acetylcholine receptor at the site of nerve-muscle contact (Ferns and Hall, 1992; McMahan, 1990), and ARIA regulates acetylcholine receptor synthesis and tyrosine phosphorylation (Falls et al., 1993; Corfas et al., 1993). Just as the postsynaptic apparatus is regulated by anterograde signals, the presynaptic apparatus is also presumed to be regulated by retrograde signals supplied from the target (Haydon and Drapeau, 1995). Our previous studies in *Helisoma* have demonstrated that muscle contact induces a presynaptic activation of protein kinase A leading to enhancement of local presynaptic calcium influx (Funte et al., 1993). Little other information, however, is available about the regulation of calcium channels at the presynaptic terminal of mammalian neurons during synaptogenesis.

The presynaptic terminal must accumulate both calcium channels and synaptic proteins at sites that are in juxtaposition to the postsynaptic receptors, for fast synaptic transmission to occur. Using antibodies directed against the α_{1b} subunit of the N-type calcium channel, we have now demonstrated that contact between pre- and postsynaptic neurons is essential for the acquisition of mature presynaptic N-type calcium channel and synaptotagmin distributions. In the absence of local instructive cues provided by a postsynaptic neuron, individual hippocampal neurons synthesize calcium channel subunits which are transported along the neurites. These subunits form functional ω-CTX GVIA-sensitive channels that permit calcium entry during depolarization. However, contact with other hippocampal neurons is required for these subunits to organize into discrete clusters. The particular signal that mediates this regulatory control is unknown. Fletcher et al. (1994) demonstrated that a property specific to " mature" dendrites is required to induce the

axonal reorganization of presynaptic vesicles in hippocampal neurons. Presumably similar signals also control the distribution of the N-type calcium channel in the axon terminals.

Westenbroek et al. (1992), using antibodies specific to α_{1b} subunits, localized N-type calcium channels in the brains of adult rats. They observed immunoreactivity on dendritic shafts and also within punctate structures located on dendrites. Reportedly, this punctate staining was particularly notable on the mossy fibers of dentate gyrus granule neurons. Using radioligand binding with conotoxin and inummoprecipitation with α_{1b} antibodies, Jones et al. (1997) demonstrated that N-type channels are expressed prenatally and are initially confined to the soma and proximal dendrites, and that punctate staining can be seen in mature neurons. Functional support for the presence of N-type calcium channels has been recently provided by whole-cell recordings performed using dendrosomes (Kavalali et al., 1997). However, the functional significance of the N-type channel in the dendrite is still uncertain. Westenbroek et al. (1992) speculated that punctate staining patterns represent N-type calcium channels in nerve terminals. Similarly, we observed that N-type calcium channel subunits are organized in punctate clusters on the neurites of synaptically mature neurons. Nevertheless, based on our data, we cannot unequivocally state that the punctate immunoreactivity of the calcium channel subunits is presynaptic. However, since the α_{1b} subunit can colocalize with the synaptic vesicle protein synaptotagmin and action potential-evoked transmitter release is reduced by ω-CTX GVIA in hippocampal cultures (Basarsky et al., 1994; Scholz and Miller, 1995), it seems likely that this immunoreactivity is due to presynaptic channels.

While the mechanisms of regulation of distribution of calcium channels and vesicles are unknown, there are many issues that now need to be resolved. For example, do calcium channels clusters at sites of neurite-neurite contact independent of synapse formation? Do calcium channels first cluster at the site of contact and act as an anchor that permits the subsequent aggregation of vesicles and synaptic apparatus? Alternatively, of course, the opposite temporal sequence may occur in which synaptic proteins first aggregate at contact sites and act as a trap for synaptic calcium channels. One might envision a role for the presynaptic plasma membrane protein neurexin in the process of aggregation of calcium channels. The neurexins, a family of proteins that have extracellular immunoglobulin motifs, are also known to bind to the vesicle protein synaptotagmin through their short cytoplasmic

tail (Ushkaryov et al., 1992). Perhaps contact with the postsynaptic target induces the clustering of neurexins in the presynaptic membrane, which in turn leads to a clustering of synaptic vesicles through the synaptotagmin-neurexin interaction. Such a complex, together with other synaptic proteins such as syntaxin, could then act as a calcium channel binding site to induce the local accumulation of N-type calcium channels.

A previous electrophysiological study has demonstrated that the development of action potential evoked transmitter release is delayed in relation to the appearance of spontaneous synaptic currents (Basarsky et al., 1994). What is the rate-limiting step in synaptogenesis between hippocampal neurons? A priori, there are four potential mechanisms that could limit the formation of synapses: the lack of (a) calcium channels, (b) secretory machinery, (c) expression of postsynaptic receptors, or (d) spatial coupling between calcium channels, secretory machinery, and postsynaptic receptors. This study has clearly ruled out the first of these possibilities, since we have demonstrated that the calcium channel subunits are synthesized and transported and form functional channels prior to synaptogenesis. In addition, the synthesis of synaptic machinery is unlikely to be rate-limiting, since synaptic proteins have been shown to be expressed prior to the appearance of action potential -evoked transmitter release (Basarsky et al., 1994). Postsynaptically, Craig et al. (1994) showed that glutamate receptors are expressed by hippocampal neurons within about 1 day of culture. Taken together, these data suggest that the limiting step in synaptogenesis is the regulation of the tight spatial coupling of molecules within and between the pre- and postsynaptic terminal.

REFERENCES

BASARSKY, T. A., PARPURA, V., and HAYDON, P. G. (1994). Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. J. Neurosci. 14:6402-6411.

BEAN, B. P. (1989). Classes of calcium channels in vertebrate cells. Annu. Rev. Physiol. 51:367-384.

BOLAND, L. M., MORRIL, J. A., and BEAN, B. P. (1994). ω-conotoxin block of N-type calcium channels in frog and rat sympathetic neurons. J. Neurosci. 14:5011 5027.

BURGOYNE, R. D. and MORGAN, A. (1995). Ca" and secretory- ves ic le dynamics. Trends Neurosci. 18:191 196.

COHEN, M. W., JONES, 0. T., and ANGELIDES, K. J. (1991). Distribution of Ca" channels on frog motor nerve terminals revealed by fluorescent ω-conotoxin. J. Neurosci. 11:1032-1039.

CORFAS, G., FALLS, D. L., and FISCHBACH, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, also induces tyrosine phosphorylation of a 185-kDa muscle transmembrane protein. Proc. Natl. Acad. Sci. USA 90:1624-1628.

CRAIG, A. M., BLACKSTONE, C. D., HUGANIR, R. L., and BANKER, G. (1994). Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitter. Proc. Natl. Acad. Sci. USA 91:12373-12377.

DOYLE, R. T., ARAQUE, A., and HAYDON, P. G. (1996). Astrocytes enhance synapse formation between cultured hippocampal neurons. Soc. Neurosci. Abstr. 22:1949.

FALLS, D. L., ROSEN, K. M., CORFAS, G., LANE, W. S., and FiSCHBACH, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell 72:801 -815.

FERNS, M. J. and HALL, Z. W. (1992). How many agrins does it take to make a synapse'? Cell 70:1 -3.

FLETCHER, T. L., DE CAMILLI, P., and BANKER, G. (1994). Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. J. Neurosci. 14:6695-6706.

FUNTE, L. R. and HAYDON, P. G. (1993). Synaptic target contact enhances presynaptic calcium influx by activating cAMP-dependent protein kinase during synaptogenesis. Neuron 10:1069-1078.

GRYNKIEWICZ, G., POENIE, M., and Tsien R Y. (1985). A new generation of Ca" indicators with greatly improved fluorescence properties. J. Biol. Chem. 3440-3450.

HAYDON, P. G. and DRAPEAU, P. (1995). From contact to connection: early events during synaptogenesis. Trends Neurosci. 18:196-200.

HAYDON, P. G., HENDERSON, E., and STANLEY, E. (1994). Localization of individual calcium channels at the release face of a presynaptic nerve terminal. Neuron 13:1275-1280.

JONES, 0. T., BERNSTEIN, G. M., JONES, E. J., JUGLOFF, D. G. M., LAW, M., WONG, W., and MILLS, L. R. (1997). N-type calcium channels in the developing rat hippocampus: subunit, complex, and regional expression. J. Neurosci. 17:6152-6164.

JONES, 0. T., KUNZE, D. L., and ANGELIDES, K. J. (1989). Localization and mobility of ω-conotoxin-sensitive Ca^{+2} channels in hippocampal CAI neurons. Science 244:1189-1193.
KAVALALI E. T., ZHUO, M., BITO, H., and Tsien R. W. (1997). Dendritic Ca'- channels characterized by recordings from isolated hippocampal dendritic segments. Neuron 18:651 -663.

LLINAS, R., SUGIMORI, M., HILLMAN, D. E., CHERSKEY, B. (1992). Distribution and functional significance of P-type, voltage-dependent Ca2+ channels in the mammalian nervous system. *Trends Neurosci.* 15:351-355.

LUEBKE, J. I., DUNLAP, K., and TURNER, T. J. (1993). Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron* 11:895-902.

MCMAHAN, U. J. (1990). The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* 55:407-418.

MILLER, R. J. (1992). Voltage-sensitive Ca . J. *Biol. Chem.* 267:1403-1406.

OLIVERA, B. M., MCINTOSH, J. M., Cruz L. J., LUQUE, F. A., and GRAY, W. R. (1984). Purification and sequence of a presynaptic peptide toxin from *Conus geographicus* venom. *Biochemistry* 23:5087-5090. ROBITAILLE, R., ADLER, E. M., and CHARLTON, M. P. (1990). Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* 5:773-779.

ROLE, L. W., MATOSSIAN, V. R., O'BRIEN, R. J., and FISCHBACH, G. D. (1985). On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. J. *Neurosci.* 5:2197-2204.

REUTER, H. (1995). Measurements of exocytosis from single presynaptic nerve terminals reveal heterogeneous inhibition by Ca2+-channel blockers. *Neuron* 14:773-779.

SCHOLTZ, K. P. and MILLER, R. J. (1991). GABAB receptor-mediated inhibition of Ca 21 currents and synaptic transmission in cultured rat hippocampal neurons. *J. Physiol.* 444:669-686.

SCHOLTZ, K. P. and MILLER, R. J. (1995). Developmental changes in presynaptic calcium channels coupled to glutamate release in cultured hippocampal neurons. J. *Neurosci.* 15:4612-4617.

TAKAHASHI, T. and MOMIYAMA, A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature* 366:156-158.

TORRI TARELLI, F., PASSAFARO, M., CLEMENTI, F., and SHER, E. (1991). Presynaptic localization of omega-conotoxin- sensitive calcium channels at the frog neuromuscular junction. *Brain Res.* 547:331-334.

TSIEN R. W., LIPSCOMBE D., MADISON, D. V., BLAY, K. R., and Fox, A. P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* 11:431-438.

TURNER, T. J., ADAMS, M. E., and DUNLAP, K. (1993). Calcium channels coupled to glutamate release identified by w-AGA-IVA. *Science* 258:310-313.

USHKARYOV, Y. A., PETRENKO, A. G., GEPPERT, M., and SUDHOF, T. C. (1992). Neurexins: synaptic cell surface proteins related to the a-latrotoxin receptor and laminin. *Science* 257:50-56.

VERDERIO, C., COCO, S., FUMAGALLI, G., and MATTEOLI, M. (1995). Calcium-dependent glutamate release during neuronal development and synaptogenesis: different involvement of omega-agatoxin IVA-and omega-conotoxin GVIA-sensitive channels. *Proc. Natl. Acad.* Sci. USA 92:6449-6453.

WESTENBROEK, R. E., HELL, J. W., WARNER, C., DUBEL, S. J., SNUTCH, T. P., and CATTERALL, W. A. (1992). Biochemical properties and subcellular distribution of an N-type calcium channel alpha I subunit. *Neuron* 9:1099-1115.

WHEELER, D. B., RANDALL, A., and Tsien R. W. (1994). Roles of N-type and Q-type Ca" channels in supporting hippocampal synaptic transmission. *Science* 264:107

WITCHER, D. R., DE WAARD, M., and CAMPBELL, K. P. (1993a). Characterization of the purified N-type Ca^{+2} channel and the cation sensitivity of omega-conotoxin GVIA binding. *Neuropharmacology* 32:1127-1139.

WITCHER, D. R., DE WAARD, M., SAKAMOTO, J., FRANZINI-ARMSTRONG, C., PRAGNELL, M., KAHL, S. D., and CAMPBELL, K. P. (1993b). Subunit identification and

reconstitution of the N-type Ca 21 channel complex purified from brain. *Science* 261:486-489.

ZORAN, M. J., DOYLE, R. T., and HAYDON, P. G. (1991). Target contact regulates the calcium responsiveness of the secretory machinery during synaptogenesis. *Neuron* 6:1-10.

GENERAL REFERENCES

Araque, A., N. Li, R.T. Doyle, and P.G. Haydon. 2000. SNARE protein-dependent glutamate release from astrocytes. *J Neurosci.* 20:666-73.

Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci.* 10:2129-42.

Araque, A., V. Parpura, R.P. Sanzgiri, and P.G. Haydon. 1999a. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22:208-15.

Araque, A., R.P. Sanzgiri, V. Parpura, and P.G. Haydon. 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J Neurosci.* 18:6822-9.

Araque, A., R.P. Sanzgiri, V. Parpura, and P.G. Haydon. 1999b. Astrocyte-induced modulation of synaptic transmission. *Can J Physiol Pharmacol.* 77:699-706.

Charles, A.C., J.E. Merrill, E.R. Dirksen, and M.J. Sanderson. 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron.* 6:983-92.

Cornell-Bell, A.H., and S.M. Finkbeiner. 1991. Ca2+ waves in astrocytes. *Cell Calcium.* 12:185-204.

Cornell-Bell, A.H., S.M. Finkbeiner, M.S. Cooper, and S.J. Smith. 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science.* 247:470-3.

Dani, J.W., A. Chernjavsky, J. Buchanan, and S.J. Smith. 1991. Neuronal activity elicits astrocyte Ca waves and oscillations within hippocampal slices. *In* Society for Neuroscience. Vol. 17. 56.

Dani, J.W., A. Chernjavsky, and S.J. Smith. 1990. Calcium waves propagate through astrocyte networks in developing hippocampal brain slices. *In* Society for Neuroscience. Vol. 16. 970.

Dani, J.W., A. Chernjavsky, and S.J. Smith. 1992. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron*. 8:429-40.

Dani, J.W., and S.J. Smith. 1995. The triggering of astrocytic calcium waves by NMDA-induced neuronal activation. *Ciba Found Symp*. 188:195-205.

Dierig, S. 1994. Extending the neuron doctrine: Carl Ludwig Schleich (1859-1922) and his reflections on neuroglia at the inception of the neural-network concept in 1894. *Trends Neurosci*. 17:449-52.

Doyle, R.T., A.A. Araque, and P.G. Haydon. 1996. Astrocytes enhance synapse formation between cultured rat hippocampal neurons. *In* Society for Neuroscience. Vol. 1996. 1949.

Eccles, J.C., and G. W.C. 1979. Sherrington, His Life and Thought. Springer-Verlag, Berlin. 269 pp.

Edwards, J.S., and R. Huntford. 1998. Fridtjof Nansen: from the neuron to the North Polar Sea. *Endeavour*. 22:76-80.

Finger, S. 2000. Minds behind the Brain: a history of the pioneers and their discoveries. Oxford University Press, New York. 364 pp.

Finkbeiner, S. 1992. Calcium waves in astrocytes-filling in the gaps. *Neuron*. 8:1101-8.

Finkbeiner, S.M. 1993. Glial calcium. *Glia*. 9:83-104.

Fodstad, H., D. Kondziolka, and A. de Lotbiniere. 2000. The Neuron Doctrine, the Mind, and the Artic. *Neurosurgery*. 47:1381-1389.

Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem*. 260:3440-50.

Haydon, P.G. 2001. Glia: Listening and Talking to the Synapse. *Nature Neuroscience*. 2:1-9.

Jeftinija, S.D., K.V. Jeftinija, and G. Stefanovic. 1997. Cultured astrocytes express proteins involved in vesicular glutamate release. *Brain Res*. 750:41-7.

Katz-Sidlow, R.J. 1998. The formulation of the neuron doctrine: the Island of Cajal. *Arch Neurol*. 55:237-40.

Laake, J.H., T.A. Slyngstad, F.M. Haug, and O.P. Ottersen. 1995. Glutamine from glial cells is essential for the maintenance of the nerve terminal pool of glutamate: immunogold evidence from hippocampal slice cultures. *J Neurochem*. 65:871-81.

Lapidot, A., and A. Gopher. 1994. Cerebral metabolic compartmentation. Estimation of glucose flux via pyruvate carboxylase/pyruvate dehydrogenase by 13C NMR isotopomer analysis of D-[U-13C]glucose metabolites. *J Biol Chem*. 269:27198-208.

MacVicar, B.A. 1984. Voltage-dependent calcium channels in glial cells. *Science*. 226:1345-7.

Nakanishi, K., Y. Okouchi, T. Ueki, K. Asai, I. Isobe, Y.Z. Eksioglu, T. Kato, Y. Hasegawa, and Y. Kuroda. 1994. Astrocytic contribution to functioning synapse formation estimated by spontaneous neuronal intracellular Ca2+ oscillations. *Brain Res*. 659:169-78.

Nedergaard, M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science*. 263:1768-71.

Newman, E.A., and K.R. Zahs. 1998. Modulation of neuronal activity by glial cells in the retina. *J Neurosci*. 18:4022-8.

Parpura, V., T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija, and P.G. Haydon. 1994. Glutamate-mediated astrocyte-neuron signalling [see comments]. *Nature*. 369:744-7.

Parpura, V., Y. Fang, T. Basarsky, R. Jahn, and P.G. Haydon. 1995a. Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP- 25 in cultured astrocytes. *FEBS Lett*. 377:489-92.

Parpura, V., and P.G. Haydon. 2000. From the cover: physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons [see comments]. *Proc Natl Acad Sci U S A*. 97:8629-34.

Parpura, V., F. Liu, K.V. Jeftinija, P.G. Haydon, and S.D. Jeftinija. 1995b. Neuroligand-evoked calcium-dependent release of excitatory amino acids from Schwann cells. *J Neurosci*. 15:5831-9.

Pfrieger, F.W., and B.B. A. 1996. Synapse formationin vitro requires a glia-derived signal. *In* Society for Neuroscience. Vol. 1996. 1949.

Pfrieger, F.W., and B.A. Barres. 1997. Synaptic efficacy enhanced by glial cells in vitro. *Science*. 277:1684-7.

Poenie, M., and R. Tsien. 1986. Fura-2: a powerful new tool for measuring and imaging [Ca2+]i in single cells. *Prog Clin Biol Res*. 210:53-6.

Poitry-Yamate, C.L., S. Poitry, and M. Tsacopoulos. 1995. Lactate released by Muller glial cells is metabolized by photoreceptors from mammalian retina. *J Neurosci.* 15:5179-91.

Sanzgiri, R.P., A. Araque, and P.G. Haydon. 1999. Prostaglandin E(2) stimulates glutamate receptor-dependent astrocyte neuromodulation in cultured hippocampal cells. *J Neurobiol.* 41:221-9.

Shepard, G.M. 1988. Neurobiology. Oxford University Press, New York. 689 pp.

Smith, S.J. 1992. Do astrocytes process neural information? *Prog Brain Res.* 94:119-36.

Smith, S.J. 1994. Neural signalling. Neuromodulatory astrocytes. *Curr Biol.* 4:807-10.

Smith, S.J. 1998. Glia help synapses form and function. *Curr Biol.* 8:R158-60.

Tsacopoulos, M., and P.J. Magistretti. 1996. Metabolic coupling between glia and neurons. *J Neurosci.* 16:877-85.

Tsien, R.Y. 1988. Fluorescence measurement and photochemical manipulation of cytosolic free calcium. *Trends Neurosci.* 11:419-24.

Tsien, R.Y., and A.T. Harootunian. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium.* 11:93-109.

Ullian, E., S.K. Sapperstein, K.S. Christopherson, and B.A. Barres. 2001. Control of Synapse Number by Glia. *Science.* 291:657-660.

Verderio, C., S. Coco, E. Pravettoni, A. Bacci, and M. Matteoli. 1999. Synaptogenesis in hippocampal cultures. *Cell Mol Life Sci.* 55:1448-62.

Vesce, S., P. Bezzi, and A. Volterra. 1999. The highly integrated dialogue between neurons and astrocytes in brain function. *Sci Prog.* 82:251-70.

Westergaard, N., U. Sonnewald, and A. Schousboe. 1995. Metabolic trafficking between neurons and astrocytes: the glutamate/glutamine cycle revisited. *Dev Neurosci.* 17:203-11.

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